Caractérisation des parasites du paludisme gestationnel et optimisation du potentiel vaccinal de VAR2CSA

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Liste des abréviations

°C: degré Celsius

ADN: acide désoxyribonucléique

AH: acide hyaluronique

AMA1: apical membrane antigen 1

ARN: acide ribonucléique

ATS: acidic terminal sequence

AU: arbitrary unit

AVS: Antigènes variants de surface

cDNA: complementary desoxyribonucleic acid (ADN complémentaire)

C4S: chondroitin-4-sulfate

CD: Cluster of differentiation (Complexe Membranaire)

CIDR: Cystein InterDomain Rich region

CHO: chinese hamster ovary

CMH: Complexe Majeur d'Histocompatibilité

CPA: Cellule(s) Présentatrice(s) d'Antigène(s)

CPDA: Citrate Phosphate Dextrose Anticoagulant

CSA: Chondroïtine Sulfate A

CSP : circumsporozoite protein

CSPG : chondroitin sulphate proteoglycans

CTA: Combinaison Thérapeutiques à base d'Artémisine

DBL: Duffy Binding Like

E. coli: Escherichia coli

EDTA: ethylene diamine tetraacetate

EI: érythrocytes infectés

ELISA: Enzyme-linked immunosorbent assay

EPCR: endothelial protein C receptor

FACS: Fluorescent Activated Cell Sorting

FPN: faible poids de naissance

G6PD: glucose-6-phosphate déshydrogénase

GAG(s): Glycosaminoglycans

GLURP: glutamate rich protein

GPI: Glycosylphosphatidylinositol

HbF: hémoglobine fœtale

HLA: Human Leukocytes Antigens

HP: Hématie parasitée

Hz: Hémozoïne

ICAM-1: Intercellular adhesion molecule-1

ID: inter domaine

IFN- γ : Interféron gamma

IgG: Immunoglobuline G

IL: Interleukine

Kb: kilobase

kDa: kilo Dalton

LSA-1: liver stage antigen – 1

MILD: Moustiquaire Imprégnée à Longue Durée d'action

MSP: merozoite surface protein

NK: Natural Killer

NTS: N-terminal segment

OMS: Organisation Mondiale de la Santé

PABA: p-aminobenzoic acid (p-aminobenzoïque acide)

PAG: Paludisme Associé à la Grossesse

PAM: pregnancy associated malaria

Pb: pair de base

PECAM-1 : platelet/endothelial cell adhesion molecule-1

PCR: polymerase chain reaction

PfEMP1 : *P. falciparum* Erythrocyte Membrane Protéine 1

PG: paludisme gestationnel

RIFIN: repetitive interspersed family

SP: Sulfadoxine Pyrimétamine

STEVOR: subtelomeric variable open reading frame

SURFIN: surface-associated interspersed gene family

TDR: Test de Diagnostic Rapide

TLR: Toll like receptor

TM: transmembranaire

TNF: Tumor necrosis factor

TPI-SP: Traitement Préventif Intermittent-Sulfadoxine Pyrimétamine

UTR: unstranslated region

VIH: virus de l'immunodéficience humaine

Liste des tableaux et figures

Liste des articles inclus dans la thèse

Article 1: Doritchamou J, Bertin G, Moussiliou A, Bigey P, Viwami F, Ezinmegnon S, et al. First-trimester *Plasmodium falciparum* infections display a typical « placental » phenotype. *J Infect Dis*. 2012; 206 (12):1911 - 1919.

Article 2: Doritchamou J, Sossou-Tchatcha S, Cottrell G, Moussiliou A, Hounton Houngbeme C, Massougbodji A, et al. Dynamics in the Cytoadherence Phenotypes of *Plasmodium falciparum* Infected Erythrocytes Isolated during Pregnancy. *PLoS ONE*. 2014;9(6):e98577.

Article 3: Bigey P, Gnidehou S, Doritchamou J, Quiviger M, Viwami F, Couturier A, et al. The NTS-DBL2X region of VAR2CSA induces cross-reactive antibodies that inhibit adhesion of several *Plasmodium falciparum* isolates to chondroitin sulfate A. *J Infect Dis*. 2011; 204(7):1125-33.

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Autres publications Durant la thèse

- Bertin GI, Lavstsen T, Guillonneau F, Doritchamou J, Wang CW, Jespersen JS, et al. Expression of the Domain Cassette 8 *Plasmodium falciparum* Erythrocyte Membrane Protein 1 Is Associated with Cerebral Malaria in Benin. *PLoS ONE*. 2013; 8(7):e68368.
- Moussiliou A, Sissinto-Savi De Tove Y, Doritchamou J, Luty AJ, Massougbodji A, Alifrangis M, et al. High rates of parasite recrudescence following intermittent preventive treatment with sulphadoxine-pyrimethamine during pregnancy in Benin. *Malar J*. 2013; 12(1):195.

- Bordbar B, Gnidehou S, Ndam NT, Doritchamou J, Moussiliou A, Quiviger M, et al. Electroporation-mediated genetic vaccination for antigen mapping: Application to *Plasmodium falciparum* VAR2CSA protein. *Bioelectrochemistry*. 2011.

Résumés

Ce travail a eu pour objectif de caractériser les parasites *P. falciparum*, infectant les femmes enceintes et responsables du paludisme associé à la grossesse.

Dans la première partie de ce travail, le phénotype d'adhérence et le profil d'expression des gènes *var* chez les parasites infectant les femmes enceintes ont été étudiés. Ces analyses ont été réalisées sur des isolats parasitaires collectés dans deux études menées au Bénin entre 2008 et 2013.

La première étude, sur des isolats prélevés dans le cadre d'une étude de cohorte de femmes enceintes dans les zones rurales au Bénin, a montré que les parasites qui infectent les femmes dans le premier trimestre de la grossesse expriment déjà un phénotype placentaire. Dans une deuxième étude, portant sur les isolats prélevés en prospective chez des femmes enceintes se présentant en consultations prénatales dans les centres de santé à Cotonou, une analyse plus détaillée des propriétés d'adhérence des hématies parasitées par *P. falciparum*, a confirmé nos premiers résultats. Cette étude a souligné une plus grande complexité dans les propriétés d'adhérence des isolats obtenus pendant le premier trimestre de grossesse qui adhèrent sur plusieurs récepteurs. Tout au long de la grossesse cette diversité phénotypique s'affine vers des phénotypes typiquement placentaires. Ce travail a également démontré, qu'au-delà de la parasitémie, l'adhérence à la CSA est un facteur important au regard de l'issue défavorable de la grossesse ; les parasites qui infectent les primigestes adhèrent en moyenne plus à la CSA que ceux provenant des multigestes. Conformément aux travaux antérieurs, les études menées au Bénin ont montré que le gène *var2csa* est le membre de la famille des gènes *var* qui est préférentiellement transcrit par les parasites infectant la femme enceinte. L'expression de la protéine correspondante à la surface des érythrocytes infectés a été démontrée grâce à des anticorps spécifiques. Cette expression a été étroitement associée à la capacité des isolats à adhérer, *in vitro*, à la CSA.

La deuxième partie de ce travail a examiné le potentiel vaccinal de VAR2CSA par l'exploration des régions peptidiques impliquées dans l'acquisition d'anticorps « anti-adhérence ». En utilisant une approche d'immunisation génétique chez la souris, nous avons été en mesure d'identifier la région minimale de VAR2CSA située dans son extrémité N-terminale comme étant le site qui concentre les épitopes anti-adhérence. Les IgG induites contre la région NTS-DBL2X de VAR2CSA ont été capables d'inhiber l'adhérence de plus de 60% des isolats naturels de *P. falciparum* à la CSA. Cette étude a par ailleurs mis en évidence

des propriétés fonctionnelles des IgG qui seraient souche-spécifique ainsi qu'a aidé à formuler une hypothèse concernant la combinaison antigénique des variants de VAR2CSA nécessaires pour garantir une activité optimale sur des isolats de terrain.

La dernière partie de ce travail a porté sur l'analyse du polymorphisme des séquences de VAR2CSA dans sa partie N-terminale chez les isolats de terrain. Ces analyses ont démontré l'existence d'une région dimorphique dans le domaine structurellement critique ID1, qui a révélé une association très intéressante avec la survenue d'infections à très forte densité de parasite.

Le travail développé dans cette thèse a permis de mettre à jour les connaissances sur les parasites qui infectent les femmes pendant la grossesse et de formuler des hypothèses sur les pistes d'optimisation moléculaire, nécessaires au développement d'un vaccin efficace à base de VAR2CSA.

This thesis aimed to characterize the *P. falciparum* parasites infecting pregnant women and causing pregnancy-associated malaria (PAM). In the first part of this work, cytoadherence phenotype and *var* genes expression profile of pregnant women parasites have been investigated on parasite isolates collected in two studies conducted in Benin between 2008 and 2013.

The first study on isolates collected as part of a cohort study of pregnant women in rural areas in Benin, showed that parasites which infect women in the first trimester of pregnancy already express placental phenotype. In a second study on isolates collected prospectively from pregnant women attending antenatal clinics in health centers in Cotonou, the analysis of the adhesion phenotype using multiple host receptors described so far confirmed the first results and highlighted a greater complexity of the binding properties in isolates collected during the first trimester and those obtained from multigravidae. This study also demonstrated that beyond parasitaemia, adhesion to CSA is a major factor for poor pregnancy outcomes. Consistent with previous reports, studies in Benin showed that *var2csa* was the most transcribed *var* gene by PAM-isolates and its surface expression on infected erythrocyte (IE) was demonstrated with specific antibodies. This was closely linked to the ability of isolates to adhere to CSA in vitro.

The second part of the work investigated the vaccine potency of VAR2CSA by exploring the region of *var2csa* involved in the acquisition of anti-adhesion antibodies. Using a DNA immunization approach performed in mice, we were able to identify the *var2csa* minimal region located in its N-terminus as the site that concentrates the anti-adhesion epitopes. The NTS-DBL2X region of VAR2CSA has been found to induce cross-reactive antibodies that inhibit adhesion of more than 60% of field *P. falciparum* isolates to CSPG. This study highlighted some strain-specific properties in functionality of the antibodies induced and helped formulate a hypothesis of antigenic FCR3 and 3D7 variants combination for optimal activity on field isolates.

The last part of this work focused on the analysis of sequence polymorphism in the N-terminal part of VAR2CSA expressed by field isolates. The analysis demonstrated the existence of a dimorphic region within the structurally critical ID1 domain that revealed a very interesting association with the occurrence of infections with very high parasite density.

The work developed in this thesis updates knowledge on parasites infecting women during pregnancy and formulates hypotheses on the molecular optimization tracks necessary for the development of an effective VAR2CSA-based vaccine.

Introduction

Selon les dernières estimations de l'Organisation Mondiale de la Santé (OMS), 207 millions de cas de paludisme auraient été enregistrés en 2012 dont 627000 ayant causé le décès (1). Parmi ces décès, 90% de cas étaient observés en Afrique dont 71% seraient des enfants âgés de moins de cinq ans (1). Outre son impact sanitaire, le paludisme impose un lourd fardeau économique à de nombreux pays endémiques, en contribuant au cycle de pauvreté et en entravant le développement économique. La forte transmission du paludisme, combinée à la pauvreté et les limites des systèmes de santé, expliquent pourquoi le paludisme reste un problème majeur de santé publique dans ces pays. Les enfants âgés de moins de cinq ans, les personnes immunodéprimées et les femmes enceintes sont les cibles les plus vulnérables. Le paludisme est une affection parasitaire causée par un protozoaire *Apicomplexa* du genre *Plasmodium*. Parmi les cinq espèces du parasite qui infectent l'homme, *Plasmodium falciparum* est la plus virulente et est responsable des formes cliniques graves et de la majorité de la mortalité associée au paludisme. L'infestation chez l'homme se fait par la piqûre d'un *Culicidae* femelle du genre *Anopheles*.

Les nombreux efforts d'élimination ou d'éradication du paludisme ont abouti à une diminution des taux d'incidence du paludisme au niveau mondial et dans la région Afrique, en 2012 (1). Ces réductions importantes sont le résultat d'une intensification des mesures et stratégies de lutte et de contrôle notamment dans la lutte anti-vectorielle, la simplification du diagnostic par l'utilisation des tests de diagnostique rapide (TDR), l'utilisation des traitements préventifs intermittents (TPI), le développement des combinaisons thérapeutiques à base d'artémisinine ou CTA et la sensibilisation et la prise en charge gratuite des cas de paludisme grave dans certains pays. Ces avancées restent toutefois fragiles, en raison du développement incessant des résistances des parasites aux antipaludiques et de l'extension progressive de la résistance des moustiques aux insecticides.

Chez la femme enceinte, le paludisme associé à la grossesse ou paludisme gestationnel (PG), est considéré comme la plus importante cause du faible poids de naissance (FPN) du nouveau-né ainsi que la cause majeure de l'anémie sévère maternelle (2). Le paludisme est responsable d'un quart des cas d'anémie sévère (3) et le FPN est la principale cause de morbidité et de mortalité dans la première année de vie (4). La prévalence de l'anémie fœtale à la naissance est élevée dans les zones d'endémie palustre et encore plus chez les nouveau-nés dont les mères sont infectées par *P. falciparum* (5). Plusieurs travaux ont montrés une différence de susceptibilité au paludisme entre les zones hypo-endémiques et les zones à forte endémicité

palustre. En zone hypo-endémique et méso-endémique, les femmes enceintes sont à nouveau sujettes à toutes les formes graves du paludisme (dont le neuropaludisme) avec des taux de mortalité 2 à 10 fois plus élevés que ceux observés chez les femmes non gravides. (6–8)

Cette forme clinique du paludisme associé à la grossesse est caractérisée par la séquestration des érythrocytes infectés (EI) par *Plasmodium falciparum*, dans le placenta. La sévérité ou non de l'infection par *P. falciparum* est en grande partie liée à la propriété de cytoadhérence acquise par les EI. Les EI isolés du placenta, expriment à leur surface, des Antigènes Variants de Surface (AVS) particuliers qui vont favoriser le tropisme placentaire (9), par l'adhérence préférentielle aux récepteurs exprimés à la surface des syncytiotrophoblastes, dont le principal est la chondroïtine sulfate A (CSA) (9–11).

Le fort taux de résistance à la chloroquine, utilisé jusqu'alors en prophylaxie du PG, a amené l'OMS à recommander dès 2004, l'usage du TPI au cours de la grossesse par la Sulfadoxine Pyriméthamine (SP) pour la prévention du paludisme. Cependant, de nombreux parasites portent des signatures moléculaires associées à la résistance à la SP et son efficacité dans le schéma actuel est chancelante, d'où la recommandation d'une 3ème dose par l'OMS (12), (13), (14). Des alternatives aux stratégies de prévention par l'utilisation d'autres molécules sont envisagées. Cependant, les antipaludiques connus pour être sans danger pour la femme et le fœtus pendant la grossesse sont rares. Face au défi permanent de la chimiorésistance, le développement d'un vaccin qui protégerait la mère et le fœtus, serait d'un grand intérêt. Une meilleure compréhension des mécanismes effecteurs spécifiques du PG devrait donc conduire à l'identification de candidats vaccins à terme. Par ailleurs, le développement de cette stratégie nécessite la caractérisation approfondie des parasites du PG. C'est une condition préalable pour aider à identifier les épitopes pouvant conduire à l'induction d'une immunité protectrice au delà des multiples sous-variants du potentiel candidat vaccin.

En s'inscrivant dans ce cadre, cette thèse vise à générer d'avantage de données sur la caractérisation moléculaire et phénotypique des parasites infectant la femme enceinte. De plus nous avons exploré le potentiel immuno-protecteur de VAR2CSA, qui a été identifié comme la protéine parasitaire majeure impliquée dans la pathogenèse du PG.

Partie I : Revue bibliographique

Chapitre 1 : Généralité sur le paludisme

1.1. Historique, espèces pathogènes et distribution géographique

Hippocrate (460 à 370 av. J-C), a été le premier à décrire les signes clinique du paludisme, notamment la fièvre intermittente qu'il avait associé à certaines conditions climatique et environnementales. Plusieurs siècles plus tard, Alphonse Laveran (en 1880) a identifié le protozoaire responsable de la maladie qui sera appelé en 1885, *Plasmodium*. Treize ans après (1898), Ronald Ross, prouvait que le paludisme est transmis par piqûre d'un moustique; l'Anophèle. Malgré les efforts d'éradication observés dans plusieurs parties du globe, environ la moitié de la population mondiale vit de nos jours dans des régions sous incidence du paludisme (Figure 1). C'est donc l'une des causes les plus importantes de morbidité et de mortalité dans les régions tropicales et subtropicales. Le paludisme chez l'homme résulte de l'infection par l'une des espèces de *Plasmodium* ci après : *Plasmodium falciparum, Plasmodium vivax*, *Plasmodium ovale Plasmodium malariae* ou *Plasmodium knowlesi.* Parmi ces espèces, *Plasmodium falciparum* et *Plasmodium vivax* sont responsables des cas de morbidité, mais la majorité des manifestations cliniques graves et les décès sont attribués à *Plasmodium falciparum* (15–17). Toutefois, quelques cas de paludisme sévère et de décès ont été associés à *P. vivax*, même si le risque d'infection sévère et les taux de létalité dus à une infection à *P. vivax* n'ont pas été fermement établis (1,18,19). Ceci nécessite plus d'investigations visant à accroitre les connaissances sur les risques de morbidité sévère et de mortalité dus à *P. vivax.* Notamment chez la femme enceinte où l'infection par *P. vivax* a été associée à des cas d'anémie sévère, d'avortement spontané, de retard de croissance intra-utérin et de faible poids de naissance (20).

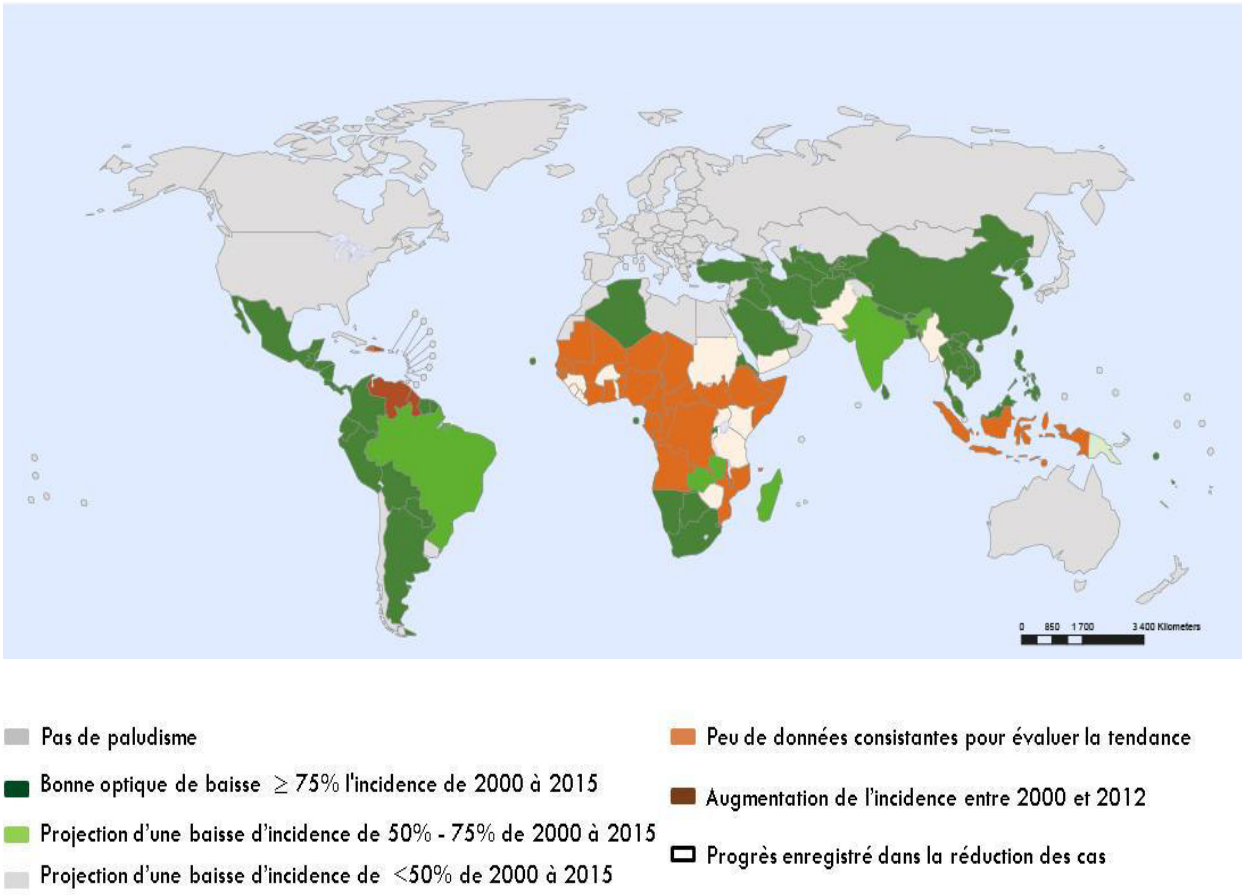

Figure 1 : Tendance de l'incidence du paludisme dans le monde (21)

1.2. Cycle biologique du *Plasmodium*

Plasmodium est un protozoaire du phylum *Apicomplexa*. Le cycle biologique du parasite, représenté dans la Figure 2, est complexe. Il est caractérisé par une reproduction asexuée chez un hôte vertébré (hôte intermédiaire), et suivi par une reproduction sexuée chez l'Anophèle femelle (hôte définitif).

- ***Chez l'anophèle***

La phase sexuée du cycle démarre après un repas sanguin sur un vertébré infecté (organisme humain) où la femelle de *Anopheles sp* ingère des gamétocytes (formes sexuées du *Plasmodium*). Dans l'estomac du moustique, les gamètes se fusionnent. Après la fécondation, il résulte un zygote diploïde appelé ookinète. Cette étape caractérise le seul stade diploïde au cours de la durée de vie du parasite. L'ookinète s'implante ensuite sous la paroi stomacale et se développe en formant l'oocyste. L'éclatement de l'oocyste libère des sporozoïtes, qui

gagnent préférentiellement les glandes salivaires du moustique (22), d'où ils pourront être injectés dans la circulation sanguine avec la salive lors d'une piqûre infectante.

- ***Chez l'homme***

Il s'effectue ici la multiplication asexuée ou schizogonique des plasmodies. Lors de la piqûre sur l'homme, le moustique infesté injecte dans le sang de l'hôte des sporozoïtes contenus dans la salive. Ces derniers regagnent rapidement le foie après un bref passage dans la circulation sanguine. Dans les hépatocytes, s'effectue la phase exo-érythrocytaire ou pré-érythrocytaire du cycle.

Phase hépatique

Au cours de cette phase, les sporozoïtes pénètrent dans les hépatocytes, grossissent avant que leurs noyaux ne se divisent par mitose pour donner des schizontes après une semaine de maturation. Cette étape du cycle du parasite est cliniquement silencieuse. L'éclatement du schizonte libère de nombreux mérozoïtes haploïdes qui passent dans la circulation sanguine pour entamer la phase intra-érythrocytaire du cycle parasitaire (23,24). Certains schizontes ou hypnozoïtes peuvent rester quiescents pendant un temps variable dans les hépatocytes (quelques mois à plusieurs années selon l'espèce) avant la libération de mérozoïtes.

Phase érythrocytaire

Dans le sang, est effectué le cycle asexué érythrocytaire (ou schizogonie érythrocytaire). Il s'accompagne des signes cliniques et des symptômes d'infection palustre. Cette phase dure 48 heures pour *Plasmodium falciparum, P. ovalae* et *P. vivax*, 24 heures pour *P. knowlesi* et 72 heures pour *P. malariae* (23) et se caractérise par la maturation et la multiplication du parasite. Les mérozoïtes pénètrent dans les hématies par endocytose et s'y transforment en trophozoïtes qui possèdent une volumineuse vacuole nutritive. Le trophozoïte, qui dégrade l'hémoglobine, grossit et sa vacuole se charge progressivement de pigments malariques ou hémozoïne (produit de la dégradation de l'hémoglobine). Son noyau se divise et chaque noyau fils s'entoure d'une plage cytoplasmique, caractéristique de la maturation du trophozoïte en schizonte mûr ou corps en rosace. Le corps en rosace, dilaté et mûr éclate, libère des mérozoïtes qui vont pouvoir parasiter de nouvelles hématies saines et perpétuer le cycle schizogonique érythrocytaire. La différenciation de certains trophozoïtes en gamétocytes (éléments à potentiel sexué mâle et femelle) intervient après plusieurs cycles schizogoniques. A maturité, les gamétocytes restent dans le sang périphérique de l'hôte intermédiaire. Ils

seront ingérés par un anophèle femelle lors d'un repas sanguin, et continueront leur développement en entamant un nouveau cycle.

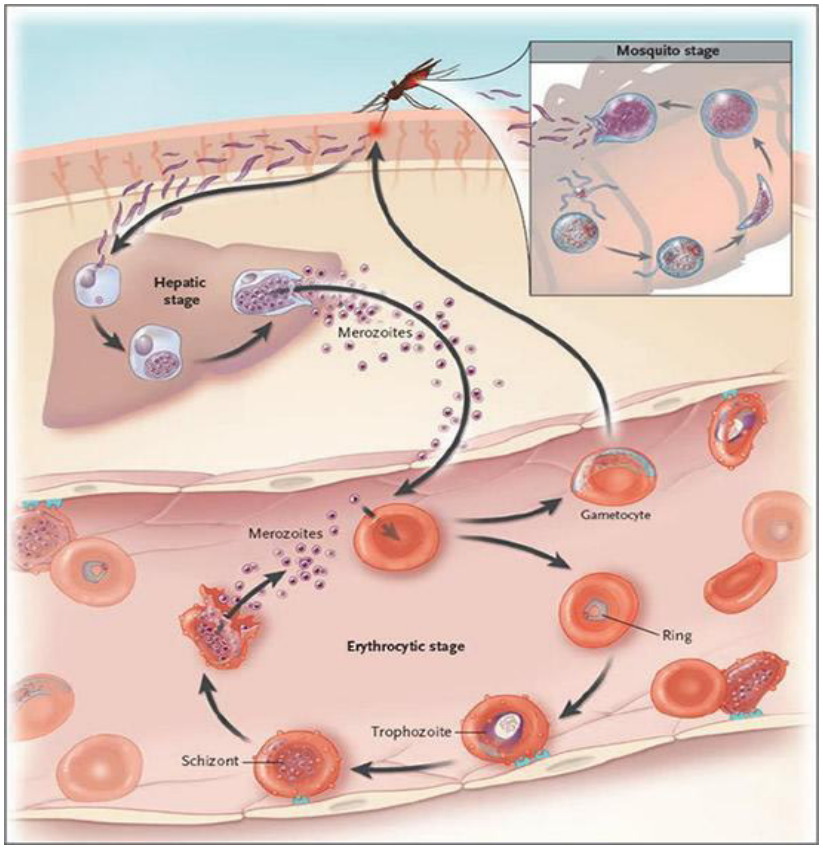

Figure 2: Cycle biologique du Plasmodium chez l'humain et le moustique. (25)

1.3. Physiopathologie du paludisme

La manifestation clinique du paludisme est étroitement associée aux différentes étapes du cycle érythrocytaire au cours desquelles plusieurs mécanismes moléculaires et immunitaires vont moduler l'infection. Ces figures cliniques varient d'une infection asymptomatique à un paludisme sévère dont la complication majeure est la mort. La lyse synchronisée des érythrocytes infectés entraine quelques symptômes cliniques tels que des maux de tête, des douleurs musculaires, de la fièvre, des frissons et de l'anémie. La fièvre serait causée par une production des cytokines pro-inflammatoires dans la circulation, en réponse aux produits de destruction des érythrocytes et des dérivés pyrogènes induits par les parasites. Les « Tumor Necrosis Factor » (TNF) sont les cytokines les plus étudiées sur ce sujet (26,27). Ces études montrent que la Glycosylphosphatidylinositol (GPI) est une protéine d'ancrage provenant de

la membrane parasitaire induisant une forte sécrétion de TNF. La baisse rapide du niveau d'hémoglobine (anémie hémolytique) au cours de la phase aigüe de l'infection serait la conséquence de l'hémolyse extravasculaire croissante concomitante à une faible production compensatrice de globules rouges par la moelle osseuse (28,29).

Plusieurs autres signes graves ont été rapportés, dont les plus fréquents sont des troubles de la conscience, des détresses respiratoires, une insuffisance rénale, des œdèmes pulmonaires, le neuropaludisme ainsi qu'une anémie sévère (30,31). Ces complications impliquent le système nerveux, les voies respiratoires, rénales et/ou hématopoïétiques. Les changements morphologiques des érythrocytes infectés les rendent susceptibles de se faire éliminer par la rate. Les parasites expriment des protéines exposées à la surface des EI. Cette altération structurale de la membrane des érythrocytes génère des protubérances ou « Knobs ». Ils sont le plus souvent caractéristiques des cellules infectées par des formes matures de *P. falciparum*. Ces changements conduisent à une rigidité accrue des érythrocytes ainsi qu'à des phénomènes d'adhérence qui peuvent entrainer des complications potentiellement mortelles. Plusieurs protéines exprimées à la surface des EI médient l'adhérence de ces derniers à plusieurs récepteurs de l'hôte évitant ainsi leur élimination par la rate. La capacité des parasites à s'accumuler dans l'endothélium vasculaire par l'intermédiaire du processus de la séquestration est la base pathogénique de la gravité du paludisme à *P. falciparum* (32). Cette séquestration résulte des interactions adhésives entre les Antigènes Variants de Surface (AVS), exprimés à la surface des EI, et des récepteurs moléculaires présents sur les cellules endothéliales, placentaires mais et au niveau des érythrocytes non-infectés (Rosetting) (Figure 3A). Ces récepteurs des AVS sont impliqués dans des syndromes cliniques particuliers du paludisme. Du fait que plusieurs organes sont des cibles de cette séquestration, plusieurs phénotypes cliniques peuvent être observés chez le malade, chacun résultant de l'organe affecté. La figure 3B présente les syndromes cliniques graves du paludisme associés à quelques organes et les types d'interactions qui caractérisent cette séquestration.

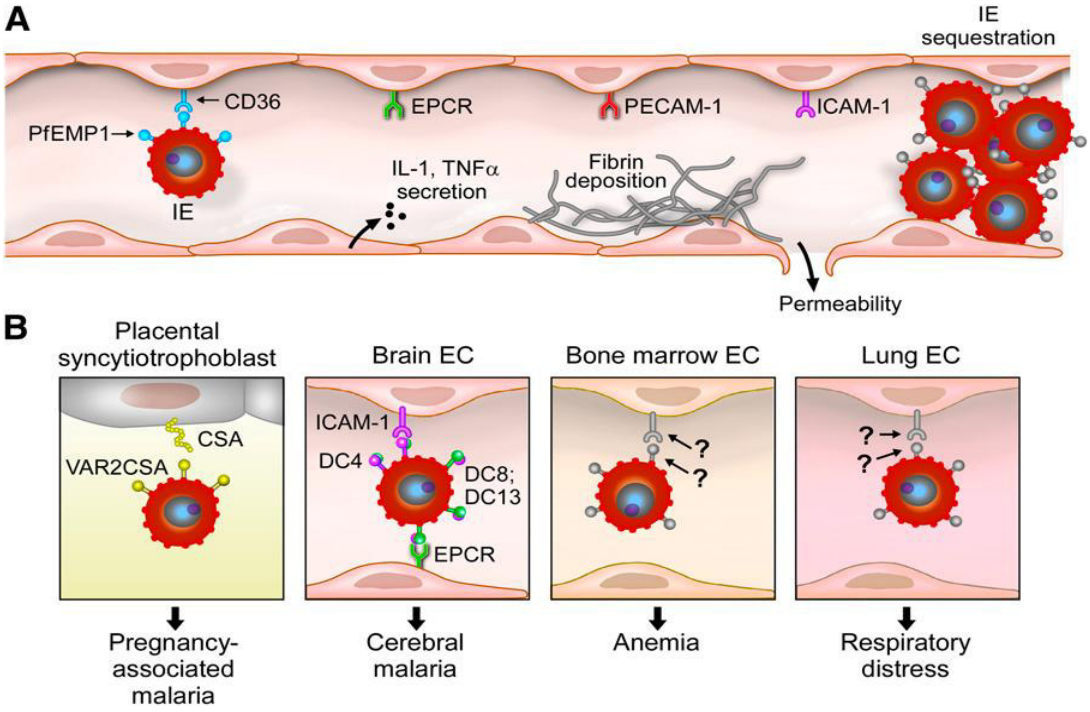

Figure 3 : Interactions caractéristiques de la séquestration des érythrocytes infectés par P. falciparum dans les micro-vaisseaux (33).

1.4. Les antigènes variant de surface

Afin d'échapper aux systèmes de défense de l'hôte contre l'infection, les parasites au cours de leur phase de développement intra-érythrocytaire se cachent à l'intérieur des érythrocytes. En effet, ceux ci sont dépourvus de CMH (Complexe Majeur d'Histocompatibilité) et donc ne sont pas reconnues par les cellules immunitaires. L'absence de ces molécules du CMH (principaux déterminants de l'acceptation ou du rejet d'une cellule du non-soi) à la surface des érythrocytes favorise le développement intra-érythrocytaire des parasites. Les parasites expriment alors à la surface des érythrocytes qu'ils infectent plusieurs protéines (34,35) dont les plus connus sont les protéines PfEMP1 (*P. falciparum* Erythrocyte Membrane Protein 1) (36–39), les protéines RIFIN (repetitive interspersed family) (40) les protéines STEVOR (subtelomeric variable open reading frame) (41,42)ainsi que les protéines SURFIN (surface-associated interspersed gene family) (43). Parmi ces AVS, la protéine PfEMP1 est le plus connu. PfEMP1 est caractéristique des facteurs majeurs de virulence du parasite et la principale cible des anticorps acquis (35,44,45). Les fonctions et le rôle des protéines RIFIN, STEVOR et SURFIN dans l'immunité acquise contre le paludisme sont encore mal élucidés bien que ces protéines pourraient être des cibles importantes pour les anticorps (46–51).

La protéine PfEMP1

PfEMP1 est la protéine médiatrice dans la formation des rosettes érythrocytaires et dans l'adhérence des EI à l'endothélium vasculaire des organes de l'hôte (52). Cette protéine (200 – 350 kDa), codée par la famille des gènes *var* (53–55), présente environ 60 copies par génome haploïde selon la souche parasitaire (56). Bien que très polymorphe, PfEMP1 montre un certain niveau de conservation dans sa structure principale constituée d'un segment N-terminal (NTS), d'un nombre variable de domaines Duffy Binding Like (DBL), d'une ou deux régions Cysteine-Rich InterDomain (CIDR), d'un domaine transmembranaire (TM) et d'un segment intracellulaire assez conservé (Acidic Terminal Segment – ATS) (57,58). Les gènes *var* sont généralement de très grande taille (6 – 13kb) et possèdent deux exons (54). Le premier code pour la région variable de liaison extracellulaire et le domaine TM, tandis que le second code pour la partie intracellulaire de la protéine. Ces gènes présentent une diversité de séquence au niveau du parasite et au sein d'un même isolat parasitaire, mais leur transcription est régulée de manière à n'avoir qu'un PfEMP1 exprimé à la surface des EI (59,60). Le processus de cette régulation transcriptionnelle des gènes *var* est encore mal élucidé. En présence d'une immunité antérieur contre un variant de PfEMP1, le système immunitaire est capable d'inhiber l'adhérence des EI. Le parasite va changer et exprimer un autre gène *var* de manière à échapper à l'immunité existante. Cette commutation de l'expression des gènes *var* à la surface des EI n'a pas seulement pour conséquence de modifier le phénotype d'adhérence des EI, mais aussi de perturber le développement des anticorps capables d'interférer avec PfEMP1. Cela contribue à une construction plus lente de l'immunité naturelle acquise contre *P. falciparum* (61). La protéine PfEMP1 est donc essentielle dans la pathogenèse du paludisme à *P. falciparum* et dans l'induction d'une immunité spécifique. Les gènes *var* peuvent être classés en 5 groupes majeurs (A – E). Cette classification est basée sur les polymorphismes observés dans les séquences des régions non-codantes *UPS* (Upstream Promoter Sequence) et des régions codantes (62–65). Plusieurs études ont montrées l'existence d'une association entre le groupe du gène *var*, exprimé à la surface des EI et la présentation clinique du paludisme. L'expression des gènes *var* des groupes A et/ou B a été associé à un paludisme simple ou sévère (66–68), tandis que l'expression de gène *var* du groupe C a été associée à un paludisme asymptomatique (68). Le gène *var1csa,* unique gène appartenant au groupe D, a été initialement associé à l'adhérence des isolats à la CSA (69,70), mais ne serait probablement pas exporté à la surface des EI (69).

Le seul membre du groupe E, *var2csa,* est relativement conservé et est associé au phénotype des parasites impliqués dans le paludisme placentaire (62,70).

Une autre classification, basée sur des blocs d'homologie d'alignement des séquences, a été réalisée à partir de la protéine entière de PfEMP1 (58). Ce travail a donné lieu à 628 blocs d'homologie couvrant environ 83% de tout PfEMP1 et il décrit les relations entre les séquences des blocs permettant l'identification de potentiels éléments fonctionnels conservés. Par ailleurs, une récente classification est issue d'un alignement des architectures de sous-types de domaines de 399 PfEMP1 définissant un ensemble de 21 domaines conservés appelées domaines cassettes (DC) (58). De récentes travaux ont montré que l'expression des PfEMP1, contenant les DC-8 et DC-13 chez les enfants, est associée à la manifestation clinique d'un paludisme sévère (71–73).

Trois membres de la famille des PfEMP1 (VAR1, VAR2CSA et VAR3) sont fortement conservés et présentent plus de 75% d'homologie sur plusieurs domaines. La plupart des autres PfEMP1 affichent eux, une identité des séquences d'acide aminé inférieur à 50% (58,74). Cependant la fonction des protéines codées par les gènes *var1* et *var3* est encore mal élucidée. VAR2CSA a été identifié comme le principal médiateur de l'adhérence des parasites au placenta au cours du paludisme gestationnel (62,75).

La protéine VAR2CSA

VAR2CSA est une protéine transmembranaire de 350 kDa. Sa partie extracellulaire est composée de 6 domaines DBL entrecoupés par des inter-domaines de taille variable et des régions TM et ATS (Figure 4). *Var2csa* a été identifié comme le seul gène surexprimé par les EI sélectionnés pour adhérer à la CSA, la protéine qui en résulte est le principal récepteur responsable de l'adhérence des EI au placenta (62,76). Il est reconnu par les anticorps présents chez les femmes enceintes infectées par *P. falciparum* de façon sexe et parité dépendante (5,77,78). Dès lors, de nombreux travaux de recherche ont visé le développement d'un vaccin à base de VAR2CSA qui empêcherait l'adhérence des EI au placenta et protègerait ainsi les femmes enceintes contre une séquestration placentaire. Ceci a fait de VAR2CSA la protéine la mieux caractérisée parmi toutes les protéines PfEMP1. Bien que relativement conservée, VAR2CSA comporte également des régions fortement polymorphes et semi-conservées dans les domaines DBL (79). Ces régions polymorphes sont exposées en surface et sous la pression immunitaire.

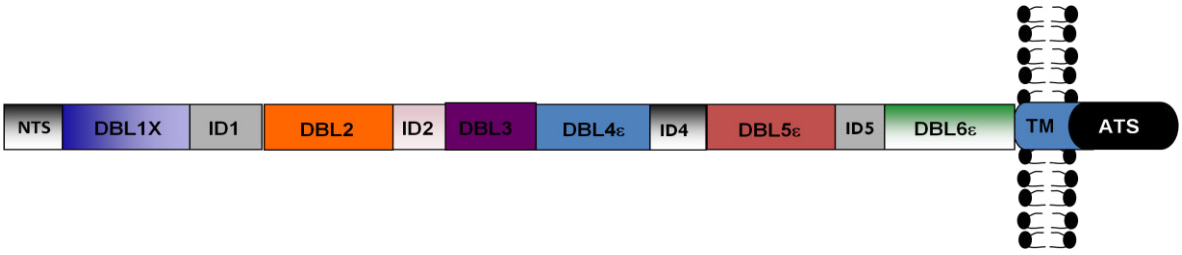

Figure 4 : Structure de VAR2CSA

1.5. Immunité et facteurs génétiques de résistance contre le paludisme

Dans les régions de forte endémicité palustre, les enfants âgés de moins de 5 ans sont les plus susceptibles à faire face à une infection palustre et ont plus de risque de présenter une forme grave comparativement aux individus adultes. Cette différence de susceptibilité repose sur l'acquisition d'une immunité clinique contre l'infection palustre à travers la construction d'un répertoire d'anticorps contre les antigènes parasitaires au cours des infections répétées durant plusieurs années (Figure 5). En zone hypo-endémique et méso-endémique, cette acquisition d'immunité contre paludisme va au-delà de 5 ans chez les enfants. Les mécanismes impliqués dans cette immunité acquise de façon progressive sont encore mal élucidés. Toutefois, la compréhension de cette immunité peut se baser sur ces observations :

- L'immunité est progressivement acquise au cours des années d'exposition à une infection palustre (31,80,81) ;
- Le transfert passif d'immunoglobulines d'un sujet adulte cliniquement immun peut guérir l'infection palustre chez un enfant (82) ;
- Des adultes cliniquement immuns restent asymptomatiques malgré une parasitémie. Cette immunité clinique acquise n'est pas stérile. Elle est fonction de la capacité du système immun à contrôler l'infection (83,84);
- Des études séro-épidémiologiques ont montré qu'un niveau élevé d'anticorps spécifiques des antigènes palustre est significativement corrélé à la protection ou à la réduction du risque d'infection palustre (85,86).

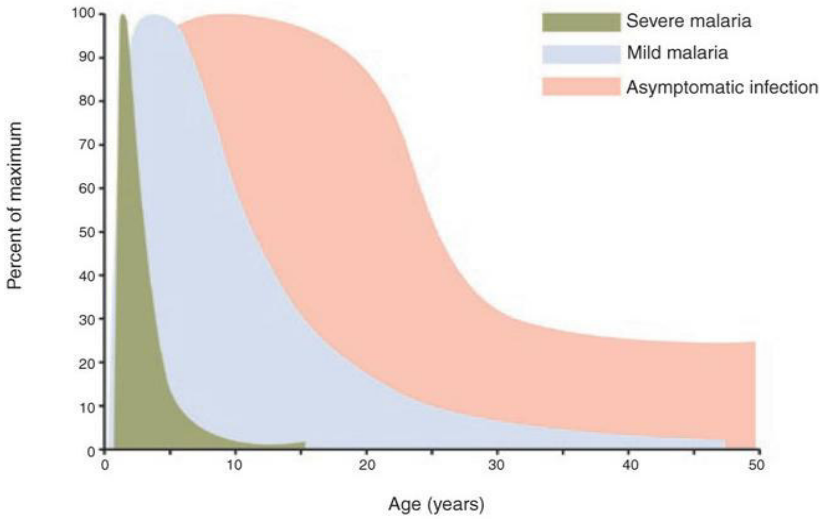

Figure 5 : Acquisition progressive d'une immunité contre P falciparum en zone de forte transmission (87)

Comme c'est le cas dans d'autres infections, l'immunité contre le paludisme est le résultat d'une combinaison de la résistance génétique, de l'immunité non-adaptatif et de l'immunité acquise ou adaptative.

1.5.1. Facteurs génétiques de résistance

Les mécanismes naturels de défense contre le paludisme sont plus apparents dans les populations exposées sans cesse à des parasites. La sensibilité aux infections palustres dépend du rôle des récepteurs de globules rouges face à l'invasion des mérozoïtes (88). En effet, les érythrocytes humains avec un déficit d'antigène Duffy de groupe sanguin (Duffy-négatif) ou qui ont eu ces antigènes enlevés par un traitement à la chymotrypsine, sont résistants à l'invasion par les mérozoïtes de *P. knowlesi* (89). D'autres études ont montrés que des individus atteints de maladies héréditaires ou présentant certains facteurs génétiques tels que l'anémie falciforme (drépanocytose) (90–92), la bêta-thalassémie (91,93), , un déficit en glucose-6-phosphate déshydrogénase (G6PD) (94) ou une ovalocytose héréditaire (95) sont protégés contre l'infection palustre.

1.5.2. Immunité naturelle acquise

Le système immunitaire inné constitue la première ligne de protection contre le parasite. Ce système de défense utilise les macrophages, les cellules dendritiques, les cellules tueuses

naturelles (NK) et les lymphocytes T (96,97). Ceux ci produisent des cytokines immunitaires afin de limiter la phase initiale de la réplication du parasite permettant à l'hôte de développer des réponses adaptatives spécifiques (98). Les nouveaux nés sont relativement protégés face aux manifestations cliniques et sévères d'une infection palustre au cours des 3 à 6 premiers mois de vie (99,100). Le mécanisme exact de cette protection est encore mal élucidé, mais la transmission des IgG maternelles dans le placenta et par le lait maternel au fœtus, l'hémoglobine fœtale (HbF), et l'absence de l'acide p-amino benzoïque dans le lait maternel ont été suggérés comme des causes possibles (101). Pour ce dernier mécanisme, l'explication se base sur la dépendance de la croissance des parasites à une source externe d'acide p-aminobenzoïque (PABA), qui est à un niveau très faible dans le lait maternel. Ainsi, l'augmentation du risque de paludisme clinique avec l'âge pourrait être associée à l'introduction des aliments de sevrage contenant PABA ou des précurseurs PABA (101). Avec l'âge et les contacts successifs entre l'humain et le parasite, il s'installe peu à peu une prémunition (symptômes atténués d'une maladie qui protège contre une infection ultérieure de type sévère). Cela fait appel à des mécanismes de résistance à l'infection parmi lesquels les protéines « interférons », métabolisées et excrétées entre autres par le foie, jouent un rôle majeur dans l'immunité anti-parasitaire. Cette immunité est dite labile car la prémunition disparait avec l'absence de contacts fréquents entre l'être humain et le parasite. L'âge et l'exposition continue au paludisme pourrait donc expliquer théoriquement cette prémunition. Ce phénomène peut s'expliquer soit par la maturation du système immunitaire au cours de la croissance de l'enfant et son passage à l'âge adulte soit par les conséquences d'une exposition cumulée durant des années d'infections répétées. Le développement d'une immunité durable est dès lors compromis en l'absence d'une exposition continue au paludisme et les individus infectés développent principalement une réponse de courte durée par la médiation d'anticorps. Ceci suggère que le maintien de cet état immun nécessite une exposition fréquente au paludisme (102).

Les travaux de McGregor et Cohen ont montré que des IgG provenant d'individus adultes immuns, par transfert passif, pouvaient réduire la parasitémie et les signes cliniques chez les enfants (103). Ceci a été confirmé plus tard par le traitement d'enfants Thai en utilisant des IgG de donneurs adultes ouest africains (82). Le mécanisme de protection induit par des anticorps dans l'immunité naturelle acquise pourrait se produire à n'importe quel stade du cycle de développement du parasite. Les observations des études de transfert passif ont montré une réduction de la parasitémie et une protection non-stérile. Ceci suggère que ces anticorps acquis par transfert passif ciblent le stade sanguin du cycle parasitaire (82). Dans

l'immunité, au stade sanguin, les hématies parasitées ne présentant pas à leur surface des protéines cellulaires présentatrices d'antigènes, la réponse humorale est le principal acteur. Les IgG sont alors considérées comme jouant un rôle crucial dans se mécanisme (103). Ces anticorps sont efficaces sur trois différents mécanismes :

- Le blocage de l'invasion des mérozoïtes (104).
- L'induction de l'opsonisation et la phagocytose (105).
- L'adhérence des antigènes de surfaces des hématies infectées aux récepteurs de l'hôte, contribuant ainsi à la clairance parasitaire (106).

Il n'est pas totalement établi lequel de ces trois mécanismes est le plus important dans le développement de l'immunité. Néanmoins, il est clair que l'immunité clinique se réalise par l'acquisition d'un répertoire d'anticorps ciblant différents AVS. L'une des principales fonctions des AVS dans l'adhérence des EI à des récepteurs spécifiques de différents tissus, est de permettre aux parasites d'échapper à l'élimination par la rate. Ceci entraine une augmentation de la parasitémie. L'ensemble de ces particularités de la réponse immunitaire contre le paludisme est à l'origine des difficultés à élaborer un vaccin. Des anticorps dirigés contre plusieurs cibles antigéniques offriraient une meilleure protection contre les manifestations cliniques du paludisme (107–110).

1.6. Stratégies de lutte

Plusieurs stratégies de lutte contre le paludisme existent aujourd'hui et sont essentiellement axées sur la prévention (chimioprophylaxie, moustiquaires imprégnées à longue durée d'action, assainissement de l'environnement domestique, etc.) ainsi que sur les traitements par combinaisons thérapeutiques à base d'artémisinine (CTA). Des travaux de recherche en vue d'un vaccin antipaludique sûr et efficace sont en cours. Des progrès récents dans ce domaine permettent d'envisager avec optimisme la possibilité de mise au point de vaccins antipaludiques.

La chimioprophylaxie

La chimioprophylaxie repose sur l'utilisation de médicaments antipaludiques dans un cadre préventif. Elle se base sur un arsenal thérapeutique de médicaments se limitant à la chloroquine, au proguanil, à l'association pyriméthamine / dapsone, à l'association proguanil /

atovaquone, à l'association chloroquine / proguanil chlorhydrate, à la méfloquine et à la doxycycline. Une variante de cette lutte est aussi appelée : Traitement Préventif Intermittent (TPI). Chez la femme enceinte, le TPI est basé sur l'utilisation de deux doses de Sulfadoxine-Pyriméthamine (TPI-SP) administrées à un mois d'intervalle, à partir du 4ème mois de grossesse.

La lutte anti-vectorielle

Basée sur l'utilisation d'insecticides domestiques, ces actions sont capables de détruire les larves et les formes adultes d'*Anopheles sp*. Mais cette méthode semble entrainer l'apparition d'anophèles résistants aux insecticides, notamment la Perméthrine (111). De ce fait, l'utilisation d'insecticides dans une zone nécessite de connaître au préalable le niveau de résistance des anophèles afin de faire un choix adéquat. Les techniques, comme la pulvérisation intra domiciliaire, l'épandage des maisons et des flasques d'eaux par des insecticides et l'utilisation des Moustiquaires Imprégnées, sont les composantes majeures de cette lutte. A ces méthodes, il faut aussi ajouter un travail face à l'insalubrité principalement à travers l'assainissement des points d'eaux stagnantes.

Les vaccins antipaludiques

Il y a actuellement plusieurs programmes de développement de vaccins ciblant des antigènes du cycle érythrocytaire. Certains de ces antigènes sont impliqués dans l'invasion des globules rouges par le parasite et sont exprimés à la surface des mérozoïtes (MSP1, MSP2, MSP3, GLURP) ou sur l'organelle apicale telle qu'AMA-1 (112). Le développement de vaccins pré-érythrocytaire pourrait potentiellement prévenir des infections subséquentes. Les antigènes ciblés pour ce type de vaccin incluent les protéines sporozoïtaires tel que la CSP (CircumSporozoite Protein) utilisée dans le candidat vaccin RTS,S (seul vaccin de deuxième génération le mieux avancé), les protéines du stade hépatique et la LSA-1 (Liver Stage Antigen – 1). Les vaccins bloquant la transmission ciblent eux les antigènes localisés à la surface des gamétocytes (Pfs) avec pour objectif d'induire des anticorps empêchant la maturation des stades sexués du parasite chez le moustique. Dans le développement de vaccin pouvant protéger la femme enceinte des conséquences graves du paludisme au cours de la grossesse, la cible est le VAR2CSA. L'objectif est d'inhiber la séquestration des EI dans le placenta.

Chapitre 2 : Paludisme chez la femme enceinte

2.1. Pathogenèse du paludisme gestationnel

Le paludisme au cours de la grossesse est l'un des problèmes majeurs de santé publique dans les pays d'endémie palustre. Le risque de paludisme (l'infection et la maladie clinique) est plus élevé chez les femmes enceintes comparativement aux femmes non enceintes, probablement en raison de changements immunologiques (113,114), et hormonaux (bien que la nature de ces derniers fasse l'objet de débat (115,116) associés à la grossesse combiné avec l'unique, capacité d'un sous-ensemble des érythrocytes infectés à séquestrer dans le placenta. Cette nouvelle susceptibilité des femmes enceintes à l'infection palustre, malgré une immunité acquise contre *Plasmodium*, a été fortement associée à l'apparition du placenta qui est à l'origine d'une immuno-modulation du système immunitaire. De ce fait, la grossesse est un état d'équilibrage immunologique dans laquelle le système immunitaire de la mère doit rester tolérant pour le fœtus et maintenir à la fois, la compétence immunitaire de défense contre les micro-organismes. Le paludisme gestationnel a été associé à une anémie maternelle sévère qui peut aboutir à la mort, l'avortement spontané, la mort du nouveau-né à la naissance et le retard de croissance du placenta et du fœtus causant un faible poids du bébé à la naissance. Ces conséquences sont le résultat de la séquestration massive des EI dans le placenta qui est à l'origine de l'inflammation du placenta et va perturber les échanges tout les échanges fœto-maternelles (117). Le faible poids de naissance est un facteur de risque de mortalité infantile. Les nouveaux nés issus de mères dont le placenta est infecté ont un risque élevé de déclarer le paludisme dans leur première année de vie (118). Le mécanisme impliqué dans cette augmentation du risque de mortalité infantile n'est pas encore complètement élucidé. Une des explications pourrait venir du fait que le paludisme placentaire entrainerait une diminution du transfert d'anticorps maternel portant ainsi atteinte à l'immunité maternelle transférée (119,120). Les facteurs maternels associés au risque de PG sont fonction de l'âge maternel, de la parité et de l'âge gestationnel. Les jeunes femmes (primipares et multipares), en particulier les adolescentes, sont plus à risque de faire un PG que les femmes plus âgées (121,122),et cela est indépendant de la parité (122,123). La parité des femmes affecte également le risque de paludisme au cours de la grossesse (6,124,125). Cette différence de susceptibilité associée à la parité a été attribué d'une part, à la faible prolifération des cellules mononucléaires du sang des primipares comparativement aux nullipares ce qui suggère une immunodépression attribuable à la grossesse (114). Cette immunodépression pourrait être plus

prononcée chez les primipares avec une altération de la production d'IL-2 (126). D'autre part, cette différence s'explique par l'acquisition d'anticorps anti-adhérence, potentiellement protecteur retrouvés chez les multipares mais qui semblent absents chez les primipares (127). Le pic de prévalence du paludisme semble se produire au cours du deuxième trimestre (13 – 16 semaines) de grossesse et diminue vers le terme (6,128). L'infection par le VIH augmente également la susceptibilité au paludisme, entraînant une infection plus fréquente et de plus forte densité ainsi qu' une perte de l'immunité relative à la parité (129). La plupart des données disponibles rapportent des infections dues à *P. falciparum* et *P. vivax*. Mais, pour ce dernier, il y a beaucoup moins d'informations comparativement à *P. falciparum*. Très peu de données sont à ce jour disponibles sur les infections causées par les autres espèces de paludisme affectant l'humain au cours de la grossesse (*P. ovale*, *P. malariae*, et *P. Knowlesi*).

2.2. Séquestration et récepteurs placentaires

Le paludisme gestationnel est caractérisé par la séquestration des EI dans les espaces inter-villeux du placenta entrainant un paludisme qualifié de placentaire. La densité parasitaire devient plus élevée dans le placenta comparativement à la densité de parasites dans le sang périphérique. Ceci suggère que le PG est causé par les EI sélectionnés pour des récepteurs majoritairement présents dans le placenta (9,52). Le tropisme des EI pour le placenta a été attribué à leur capacité d'adhérence à la CSA. La CSA est un GlycosAminoGlycane (GAG) dont l'unité de base est l'acide glucuronique β1-3 N-acétyl galactosamine 4 sulfate. Les GAG se répartissent en cinq types principaux : l'acide hyaluronique (AH), la chondroïtine sulfate, l'héparane sulfate, le dermatane sulfate et le kératane sulfate. Ils sont présents dans plusieurs tissus et organes (le cartilage, l'os, le derme, la cornée, le poumon, le foie, le tendon, le placenta, … etc.) où ils forment des composants de la matrice extracellulaire. La fonction biologique des GAGs est très diverse et inclue le contrôle de la fixation et la migration cellulaire, la fibrinogenèse et la signalisation cellulaire (130). Plusieurs micro-organismes pathogènes utilisent les GAGs comme récepteur pour des invasions cellulaires ou tissulaires, causant l'infection (131–133). Dans le cas du paludisme gestationnel, des protéoglycanes à chondroïtine sulfate (CSPG) extracellulaires, faibles en sulfate, ont été localisés dans l'espace intervilleux du syncithiotrophoblaste et ont été identifiés comme le récepteur majeur des EI (134) dans le placenta. Une récente étude a montré que le CSPG est impliqué dans les tout premiers stades de la différenciation des trophoblastes extra-villeux humaine et est un régulateur important de placentation (spécifiquement prolifération et la migration / invasion)

(135). Les chaines Chondroitine-4-Sulfate (C4S) du CSPG sont spécifiquement impliquées dans l'adhérence des EI au placenta. Une adhérence optimale implique le motif dodecasaccharide C4S comprenant quatre disaccharides sulfatés et quatre non sulfatés (136).

Des études de caractérisation des EI placentaires et de leurs phénotypes d'adhérence ont été réalisées. Il ressort que l'existence de récepteurs supplémentaires n'est pas exclue (9,10,137). Plusieurs autres récepteurs, en plus du CSA, ont été proposés comme médiateurs de la séquestration des EI dans le placenta (138). Ceci est le thème d'une grande controverse et l'implication des ces récepteurs ne peut être exclue, ni confirmée. L'AH, les GAGs non sulfaté, les immunoglobulines non-immunes, ICAM-1 et le récepteur Fcγ néonatale ont reçu une attention particulière à cet effet (137–140). Mais l'absence de données évidentes pèse lourdement contre un rôle important pour l'un de ces récepteurs dans la séquestration placentaire des IE (64,140,141). Bien que l'adhésion des EI placentaires à CD36 (un phénotype commun aux isolats provenant de donneurs non enceintes) a été testé, il a été exclu et ne peut être considéré comme un récepteur placentaire (9). Il a été présenté comme un phénotype mineur parmi les isolats placentaires dans certaines études (10,142). La plupart des isolats placentaires CD36-adhérents contenaient également des EI CSA-adhérents, ce qui suppose que ces isolats incluaient plusieurs clones avec des phénotypes distincts de cytoadhérence qui amènent les isolats à adhérer à la CSA dans le placenta et au CD36 dans d'autres organes (10,108,142,143). Toutefois, cette adhérence *in vitro* au CD36 serait moins pertinente en termes de séquestration placentaire du fait que CD36 semble ne pas être exprimé par les syncytiotrophoblastes (139).

L'interaction VAR2CSA – CSA

Plusieurs systèmes d'expression protéique ont été utilisés pour produire des protéines recombinantes correspondant à chaque domaine DBL de VAR2CSA et leur capacité d'adhérence à la CSA a été évaluée. Parmi les 6 domaines DBL, 4 (DBL2X, DBL3X, DBL5ε et DBL6ε) adhèrent *in vitro* à la CSA (144–147). L'expression très récente de la totalité de la partie extracellulaire de VAR2CSA par deux équipes a révélé une très grande affinité et spécificité d'adhérence à la CSA, plus de mille fois supérieure à celle des domaines simples synthétisés précédemment (148,149). Cette percée capitale a offert de nouvelles opportunités dans la définition des régions de VAR2CSA qui adhèrent à la CSA. Bien que la structure cristallographique de cette protéine entière (Full-length VAR2CSA = FV2) n'est pas encore disponible, celle de 2 domaines DBL (DBL3X et DBL6ε) existe déjà et confirme les modèles

de structure proposés pour chaque DBL (146,150,151). Il a donc été suggéré que la conformation générale des domaines DBL est relativement conservée, et que ces domaines peuvent agir en tant que blocs pour former des sites de liaison (152–154). La région minimale d'adhérence à la CSA a été définie dans la partie N-terminale de VAR2CSA et le site de liaison de base se situe dans le domaine DBL2X et des parties des régions ID (155,156).

2.3. Immunité spécifique du paludisme placentaire

Malgré l'immunité acquise contre *P. falciparum*, les femmes deviennent à nouveau susceptibles d'être infectées au cours de leur grossesse. Cette susceptibilité est plus élevée chez les femmes primipares (première grossesse) dû à l'absence d'anticorps capable d'empêcher l'adhérence des EI au placenta (6,76). Les parasites infectant la femme au cours de la grossesse expriment à la surface des EI un nouvel antigène qui n'a pas encore été rencontré par celle-ci. Une diminution de cette susceptibilité chez les femmes primigestes, s'observe avec l'augmentation du nombre de grossesses et s'explique par l'acquisition d'une immunité spécifique contre le paludisme gestationnel qui est associée au développement d'une large réponse anticorps protectrice contre les EI placentaires (157). Ces anticorps reconnaissent spécifiquement VAR2CSA (l'AVS associé à la séquestration placentaire) et inhibent l'adhérence des parasites à la CSA. Les femmes enceintes infectées développent donc des anticorps anti-adhérence spécifiques (anti-VAR2CSA) qui sont associés à la protection contre le paludisme lors des grossesses ultérieures (158,159). La différence dans la susceptibilité au PG, entre les femmes enceintes primipares et multipares, est donc attribuée au déficit d'anticorps dirigés contre ce phénotype particulier des AVS chez les primipares. Des plasmas issus d'individus vivant en zones d'endémie palustre reconnaissent VAR2CSA selon le mode sexe et parité-dépendance et les anticorps responsables de cette reconnaissance sont acquis pendant la grossesse (77,78).

Certaines femmes possèdent des IgG spécifiques qui agglutinent les parasites CSA-adhérents mais qui n'empêchent pas leur adhérence à la CSA (160). De même, plusieurs études ont montré que la plupart des infections placentaires sont polyclonales (70,161,162) et donc que les femmes sont exposées à plusieurs variants de parasites placentaires au cours d'une grossesse. L'étude de la cinétique de ces anticorps montre que la plupart des primipares développent des anticorps anti-adhérence autour de vingt-quatre semaines de grossesse tandis que les multipares les développent plus tôt (12 semaines), du fait de la mémoire immunitaire

et des infections précédentes (163). Cette mémoire immunologique est caractérisée par des réponses cellulaires à travers une production élevée d'IL-2 et d'IL-4 spécifiques des souches parasitaires CSA-adhérents (164). Mais aussi caractérisé par des réponses humorales spécifiques impliquant des niveaux élevés d'IgG inhibiteur de l'adhérence des EI au placenta (78).

Le niveau d'anticorps anti-VAR2CSA baisse rapidement après le premier accouchement (165). Toutefois, l'immunité spécifique contre VAR2CSA est acquise rapidement après une nouvelle exposition aux parasites séquestrant dans le placenta au cours de la grossesse suivante (163). Les femmes multigestes ont généralement des niveaux élevés d'IgG spécifique anti-VAR2CSA et sont en grande partie à l'abri d'une parasitémie placentaire préjudiciable (10,157,166,167). Ainsi la parité dépendance du taux d'anticorps spécifiques anti-VAR2CSA et la résistance au PG sont des variables corrélées (77,167). Les anticorps anti-adhérence sont de loin la réponse immunitaire la plus étudiée. Ces anticorps semblent expliquer le mécanisme de cytoadhérence qui caractérise le PG, même si l'infection placentaire est souvent accompagnée d'infiltration de monocytes et de macrophages indiquant ainsi que l'opsonisation et la phagocytose des EI, qui jouent aussi un rôle important. *P. falciparum* induit principalement l'activité des cytokines de type TH1 avec l'augmentation relative des TNF-α et une diminution des réponses IL-10 (164). L'acquisition rapide de l'immunité suivant les grossesses successives indique, par ailleurs, que la protéine responsable de la séquestration placentaire est relativement conservée.

Chapitre 3 : Le vaccin à base de VAR2CSA

Du fait des conséquences du PG qui peuvent être fatales aussi bien pour la mère que pour le fœtus, le besoin d'un vaccin qui protègerait des complications graves de l'infection placentaire est urgent. Plusieurs approches dans le but de comprendre tous les facteurs qui interviennent dans la réponse immune protectrice anti-PG sont en cours d'investigation. Une composante majeure de la réponse antipaludique (anticorps contre la surface des EI) a été intensément étudiée dans le cas du PG, la plupart de ces études étant focalisé sur VAR2CSA. Les caractéristiques qui font de VAR2CSA une cible vaccinale pour prévenir le paludisme placentaire sont les suivantes :

- VAR2CSA est reconnu par les anticorps des individus vivants en zone d'endémie de façon sexe et parité dépendante (5,77,78,127,166) ().
- Les isolats de *P. falciparum* provenant de placentas infectés transcrivent fortement *var2csa* (70,168).
- L'expression de la protéine à la surface des EI a été détectée sur des isolats de *P. falciparum* de laboratoire sélectionnés pour adhérer à la CSA tandis qu'elle est absente à la surface des isolats ne présentant pas un phénotype d'adhérence à la CSA (77,169).
- Les séquences d'acides aminés relativement conservés (54 – 95%) (65,168,170).
- Des anticorps dirigés contre VAR2CSA ont été associés à une protection contre le paludisme gestationnel (77,78,158,159).
- La suppression partielle ou totale de *var2csa* entraine une perte de la fonction d'adhérence des parasites à la CSA (75,168).

L'objectif d'un tel vaccin anti-adhérence est d'induire des anticorps capables d'inhiber l'adhérence des EI au placenta. Une réponse anti-adhérence pouvant prévenir la séquestration favorisera une élimination des EI non-adhérents par la rate, prévenant ainsi la forte parasitémie et les symptômes cliniques graves comme une réponse inflammatoire dans le placenta. Dans le but de concevoir ce vaccin recombinant, basé sur VAR2CSA, la compréhension des mécanismes moléculaires impliqués dans les interactions entre VAR2CSA et les récepteurs placentaires est nécessaire. Ceci permettra de diriger la réponse immunitaire contre les épitopes spécifiques de l'adhérence placentaire des EI. Les défis majeurs avec cet antigène résident dans le polymorphisme des séquences protéiques de VAR2CSA et les

variants et épitopes immunogènes qui devraient être inclus dans un vaccin pour avoir une efficacité optimale.

3.1. Vaccin basé sur la partie extracellulaire de VAR2CSA (FV2)

La production en 2010 pour la première fois de toute la partie extracellulaire de VAR2CSA (FV2) et la vaccination d'animaux avec FV2 a permit d'obtenir des anticorps « anti-adhérents » qui inhibent complètement l'adhérence des parasites à la CSA (149,171). Ces anticorps inhibent des souches homologues (à fond génétique similaire au variant parasitaire utilisé pour induire les anticorps), mais pas les souches hétérologues (à fond génétique différent). Ceci suggère que les épitopes parasitaires impliqués dans l'adhérence ne sont pas identiques sur différentes souches parasitaires ou que d'autres épitopes immuno-dominants dirigent l'induction des anticorps contre des épitopes non-inhibiteurs. Du fait de la grande taille moléculaire de FV2 et l'absence d'anticorps anti-adhérence capable de transcender les souches hétérologues. Le FV2 n'est pas la construction optimale pour le vaccin.

3.2. Vaccin basé sur un seul domaine DBL de VAR2CSA

Des anticorps monoclonaux dirigés contre des domaines DBL de VAR2CSA peuvent inhiber l'adhérence des isolats placentaires à la CSA (73). DBL5ε induit chez la souris des anticorps qui reconnaissent des isolats placentaires, mais pas des isolats d'enfants. La présence d'épitopes universels soutient donc DBL5ε comme une intéressante composante de VAR2CSA à considérer pour le développement d'un vaccin (172,173). Certains motifs des séquences protéiques du domaine DBL3X de VAR2CSA étaient plus observés chez les parasites isolés des primipares comparativement aux multipares et *vice versa* (147). Ceci suggère que ces motifs antigéniques de DBL3X pourraient être associés à des propriétés biologiques parasitaires en avantages ou non chez les femmes selon leur parité. Des anticorps dirigés contre le domaine DBL4ε de VAR2CSA sont capables d'inhiber l'adhérence à la CSA (174), malgré que ce domaine de VAR2CSA n'est pas impliqué dans l'adhérence à la CSA (145). Cette surprenante observation suggère que la réponse inhibitrice capable de bloquer l'adhérence du parasite est très probablement due à un encombrement stérique. Les anticorps dirigés contre un seul domaine de VAR2CSA agiraient donc en empêchant la formation de structures conformationnelles de VAR2CSA nécessaire pour former la poche de liaison à la CSA ou par l'encombrement stérique des anticorps anti-adhérence pour cibler leurs épitopes

spécifiques. Les domaines DBL1X (175) et DBL6ε (176–178), ont également été la cible d'anticorps inhibiteurs.

3.3. Vaccin basé sur des constructions de domaines de VAR2CSA

Des IgG obtenues à partir des protéines recombinantes de DBL4ε-ID4 inhibent l'adhérence des EI, autant que les sérums immuns de femmes multigestes vivant en zones de forte transmission palustre (179). L'identification de la région N-terminal de VAR2CSA comme étant la partie spécifique de VAR2CSA impliquée dans l'adhérence à la CSA a ouvert de nouveaux axes d'investigation sur le potentiel inhibiteur de cette région (155,180). En ciblant les fragments N-terminaux on induit des anticorps inhibiteurs de l'adhérence des EI à la CSA. Avec les anticorps dirigés contre les constructions ID1-DBL2Xa et ID1-ID2a (156), on bloque efficacement l'adhérence du parasite au CSPG au même titre que les anticorps dirigés contre le domaine extracellulaire entier de VAR2CSA. Ces travaux montrent clairement que la région tronquée N-terminale de VAR2CSA est une cible majeure de la réponse immunitaire anti-adhérence dans le paludisme associée à la grossesse, et de ce fait, est un candidat vaccin convaincant.

Partie II : Problématique et objectifs de la thèse

Chez la femme enceinte, les infections parasitaires sont très fréquentes et les densités parasitaires sont plus élevées au niveau du placenta que dans le sang périphérique (5). Il est clairement admis aujourd'hui que l'accumulation des EI dans le placenta est le phénomène central dans la pathogenèse du paludisme associé à la maternité (9). Toutefois, l'ensemble des mécanismes biologiques impliqués dans ce phénomène de séquestration placentaire reste encore mal élucidé. Bien que la CSA ait été identifié comme le principal récepteur de l'adhérence des IE dans le placenta, l'implication d'autres types d'interactions ligands parasitaires / récepteurs placentaires n'est pas totalement exclue. De plus, certaines études ont montré la possibilité d'adhérence des isolats placentaires à d'autres récepteurs endothéliaux (137,181). La caractérisation plus approfondies des phénotypes d'adhérence des parasites infectant les femmes enceintes aiderait à mieux comprendre la dynamique d'établissement du phénotype d'adhérence à la CSA au cours de la grossesse.

De nombreuses équipes ont montré que des anticorps de type IgG capables d'empêcher l'adhérence des hématies parasitées au récepteur placentaire CSA sont les médiateurs de l'immunité protectrice acquise par les femmes multigestes contre le paludisme placentaire (10,11). L'identification des composantes antigéniques qui seraient capables d'induire de tels anticorps a ainsi été reconnue comme un objectif majeur à atteindre. Dès lors des études de caractérisation des parasites en vue d'identifier les AVS se sont vues accélérées avec la publication du génome complet de *P. falciparum*. La stratégie vaccinale qui est de plus en plus envisagée pour prévenir du PG est celle consistant à recréer l'immunité protectrice présente chez les multipares, ce qui empêcherait l'adhérence des hématies parasitées au placenta. La partie extracellulaire de VAR2CSA a révélé une très grande affinité et spécificité d'adhérence à la CSA et des anticorps induits contre cette protéine recombinante sont capables de bloquer l'adhérence des souches homologue à la CSA (149,171). Mais le polymorphisme important de cette protéine, sa grande taille et sa structure très complexe, représente des obstacles à franchir dans la mise au point du vaccin. La caractérisation des réponses immunitaires contre VAR2CSA combinée à des études cliniques prospectives sont désormais une condition préalable pour aider à consolider le potentiel vaccinal de cet antigène dans l'acquisition de l'immunité protectrice observée chez les multigestes. La probable nécessité de combiner un certain nombre, certainement réduit, de variants dans une formulation capable d'induire des anticorps avec un large spectre d'activité, a motivé des travaux visant à rechercher des fragments plus courts de la protéine possédant des épitopes fonctionnelles et des variants majeurs à considérer.

Cette thèse s'inscrit dans la thématique de ces travaux et vise principalement à caractériser les parasites infectant les femmes enceintes et à étudier le polymorphisme de la région attractive N-terminal de VAR2CSA où serait localisé le site de fixation à la CSA. Le but étant d'identifier les variants majeurs à considérer dans le développement vaccinal.

Cette thèse se construit autour de différentes composantes énoncées ci-dessous.

Composante 1 : Il est clairement établi que l'expression d'un groupe ou sous-groupe particulier de gène *var* a un rôle dans la virulence du parasite. Dans le cadre du PG, il est indispensable d'analyser le profile d'expression de ces gène *var* et en particulier celui de *var2csa*. Cette analyse, combinée au moment de survenue de l'infection au cours de la grossesse, devrait permettre d'identifier des périodes favorables ou non à l'infestation par des parasites exprimant préférentiellement ce gène *var* particulier. A cet effet, nous avons recueilli et caractérisé des isolats de *P. falciparum* de femmes enceintes et comparé les niveaux d'expressions de *var2csa* à ceux des groupes de personnes présentant différents syndromes de paludisme dans le sud du Bénin (Article 1)

Composante 2 : La CSA a été identifié comme étant le principal, sinon le seul, récepteur de l'adhérence des EI dans le placenta. Toutefois certains parasites collectés chez des femmes gestantes pouvaient adhérer *in vitro* à d'autres récepteurs (HA, CD36, ICAM-1). Bien que le niveau d'adhérence à ces récepteurs soit relativement faible, la possible implication de ces molécules dans le phénomène de séquestration placentaire n'est pas à écarter, surtout celles qui sont exprimées par le syncytiotrophoblaste (139). Au cours de cette étude, nous avons analysé le profile d'adhérence des EI collectés chez les femmes enceintes tout au long de la grossesse sur les trois principaux récepteurs (CSA, CD36 et ICAM-1) et évalué certains facteurs de l'hôte qui pourraient influencer le phénotype d'adhérence des parasites (Article 2)

Composante 3 : La justification de l'élaboration d'un vaccin efficace à base de VAR2CSA contre le paludisme placentaire aujourd'hui s'articule autour de la définition des zones de VAR2CSA contenant des épitopes fonctionnellement importants qui transcenderaient la diversité inter-clonale. Les études d'analyse de séquences ont montré que VAR2CSA est une protéine polymorphe constituée de zones à très fort polymorphisme inter-clonal alternées de blocs conservés. Les développements récents ont clairement démontré qu'un seul DBL ne possède pas la capacité d'induire des anticorps anti-adhérence parce que, probablement, l'expression des épitopes fonctionnels de VAR2CSA nécessite une organisation architecturale

particulière mettant en œuvre plusieurs domaines DBL. Des données convaincantes sur l'induction d'anticorps anti-adhérence ont certes été obtenues par la vaccination avec la totalité de la partie extracellulaire de VAR2CSA, mais l'utilisation d'une protéine de cette taille (350 kDA) n'est pas envisageable dans une perspective pratique de développement vaccinal. Dans ce travail, plusieurs troncatures de VAR2CSA ont été générées à partir d'un variant parasitaire. Les anticorps induits contre ces constructions ont été analysés pour identifier la région minimale permettant d'induire une réponse immunitaire « anti-adhérence », qui seraient ainsi potentiellement protectrice (Article 3).

Composante 4: Le travail précédent a permis d'identifier la construction correspondant au fragment NTS-DBL2X de l'extrémité N-terminal de VAR2CSA comme étant essentielle à une activité fonctionnelle de la protéine. Les travaux d'optimisation de cette construction ont été réalisés en affinant davantage cette troncature, afin de parvenir à une construction minimale qui conserve les épitopes anti-adhérence. Ce développement a fait l'objet d'une publication (Article 4).

Composante 5 : Comparé aux autres gènes *var*, *var2csa* est exceptionnellement conservé et affiche une homologie de séquence relativement élevée entre les souches de parasites. Cependant, la variation des séquences interclonale de *var2csa* reste tout de même élevée et la diversité de ce gène a été estimée à ~ 500 fois plus élevée au sein de la population par rapport à un ensemble aléatoire de 200 gènes typiques de *P. falciparum* (65). Ce niveau élevé de diversité est un défi crucial pour le développement d'un vaccin contre le PG qui nécessiterait la prise en compte d'un certain nombre de variants dans un candidat vaccin pour pouvoir assurer une activité large sur des populations de *P. falciparum* en circulation. Dans cette thèse, nous avons analysé la fonctionnalité des anticorps induit contre la construction d'intérêt (NTS-DBL2X) produit à partir de deux variants parasitaires et défini certains variants à considérer dans une stratégie d'optimisation vaccinale (Article 5)

Composante 6 : Une analyse quantitative et qualitative des anticorps acquis naturellement contre ces cibles antigéniques de VAR2CSA, sur des échantillons provenant de sites géographiquement éloigné caractérisés par un profile épidémiologique de transmission du paludisme différent, aiderait à mieux comprendre la dynamique d'acquisition des anticorps dirigés contre VAR2CSA (Article 6).

Composante 7 : Dès lors, il apparaît que les constructions tronquées de *var2csa*, composées de fragments courts comme ID1-DL2X, représentent des candidats les plus prometteurs pour le développement d'un vaccin efficace contre le PG. Une analyse plus approfondie de la diversité fonctionnelle dans cette région attractive de VAR2CSA, contenant les épitopes anti-adhérence, est nécessaire pour guider le développement de ce vaccin. En particulier, l'évaluation de l'existence des différences dans le répertoire des allèles de *var2csa* pour les régions d'intérêt. Le but étant de déterminer si des variants spécifiques supplémentaires doivent être pris en compte. Une composante majeure de ce travail de thèse a permis d'approfondir ces analyses moléculaires ainsi que la génétique des populations de *P. falciparum* pour cette région du gène au sein de parasites collectés dans le sud du Bénin (Données en cours de publication)

Partie III : Résultats

Article I: First-trimester Plasmodium falciparum infections display a typical "placental" phenotype.

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La capacité des hématies parasitées par *Pf* à séquestrer dans le placenta est la caractéristique majeure du paludisme associé à la grossesse. Le TPI administré à la femme au cours de la grossesse présente une insuffisance majeure liée à sa couverture partielle de la grossesse. En effet, le premier trimestre de la grossesse n'est pas concerné par cette stratégie de prévention, d'une part parce que le taux de fréquentation des cliniques prénatales par les femmes dans leur premier trimestre de grossesse est généralement faible et d'autre part pour des raisons de sécurité liées au potentiel risque tératogène des molécules utilisées pendant le premier trimestre de grossesse. Les infections à *Pf* survenant en début de grossesse méritent donc des études plus détaillées sur leur contribution à la morbidité maternelle et leurs conséquences sur le déroulement de la grossesse. Quelques études ont pu montrer une association entre la parasitémie observée dans le premier trimestre de grossesse et l'issue défavorable de la grossesse, à la fois en termes de poids de naissance et d'anémie maternelle. Ceci souligne le potentiel risque auquel la mère et le fœtus sont exposés en début de gestation. Par ailleurs, il est clairement établi que la virulence du parasite est liée à l'expression différentielle d'un groupe ou sous-groupe particulier de gène *var* par le parasite.

Ce travail avait pour objectif de caractériser les phénotypes des parasites provenant de 91 femmes incluses à différents moments de la grossesse (dont 41 femmes à l'accouchement). Les niveaux de transcription des gènes *var* ont été mesurés et comparé avec ceux des parasites obtenus de patients souffrant de paludisme simple et d'enfants présentant un paludisme cérébral. Le niveau d'expression de VAR2CSA à la surface des EI et leurs propriétés d'adhérence au CSPG ont également été évalués.

Des amorces spécifiques des différents groupes et sous-groupes de gènes *var* ont été utilisées pour amplifier des cDNA par qPCR, afin de quantifier le nombre de copies de ces gènes et analyser leur profil transcriptionnel. Des IgG anti-VAR2CSA, produites chez le lapin à partir

de la séquence de la souche FCR3, ont été utilisées pour analyser l'expression de VAR2CSA en surface des EI par cytométrie en flux. Cet outil a également servi à évaluer la séroréactivité des plasmas d'individus donneurs de parasites sur des lignées contrôles sélectionnés pour présenter un phénotype d'adhérence à la CSA. Les niveaux plasmatiques d'anticorps dirigés contre les antigènes AMA1 et les domaines DBL3X et DBL5ε de VAR2CSA ont également été mesurés par ELISA.

La quantification des transcripts des gènes *var* montre clairement une différence entre les parasites obtenus des différents groupes cliniques étudiés. Il en ressort que les parasites provenant des femmes enceintes transcrivent préférentiellement *var2csa* tandis que ceux isolés des enfants présentant un neuro-paludisme transcrivent fortement les gènes *var* du groupe A. Les données sur les niveaux d'expression de VAR2CSA en surface des EI confortent ces observations et mettent d'avantage l'accent sur les caractéristiques phénotypiques particulières des parasites qui infectent les femmes enceintes. En s'intéressant au groupe des femmes enceintes, nous avons constaté que les parasites transcrivaient *var2csa* à des niveaux élevés indépendamment de l'âge gestationnel des femmes. De plus la capacité d'adhérence au CSPG de ces isolats ne montre pas de différence significative entre les isolats collectés dans le premier trimestre de grossesse et ceux collectés tard dans la grossesse ou à l'accouchement.

Ce travail est le premier à montrer que le tropisme placentaire de *P. falciparum* est déjà établi dès le premier trimestre de la grossesse avec des parasites qui expriment des phénotypes typiquement placentaires. Nos résultats confirment la particularité de l'expression de VAR2CSA dans la pathogenèse du paludisme associé à la grossesse et apportent d'avantage d'arguments en faveur de son utilisation comme candidat majeur pour le développement d'un potentiel vaccin pour prévenir du paludisme placentaire.

First-Trimester *Plasmodium falciparum* Infections Display a Typical "Placental" Phenotype

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Background. *Plasmodium falciparum*–infected erythrocytes (IEs) adhere to host cell receptors, allowing parasites to sequester into deep vascular beds of various organs. This defining phenomenon of malaria pathogenesis is key to the severe clinical complications associated with cerebral and placental malaria. The principal ligand associated with the binding to chondroitin sulfate A (CSA) that allows placental sequestration of IEs is a *P. falciparum* erythrocyte membrane protein 1 (P*f*EMP1) family member encoded by the *var2csa* gene.

Methods. Here, we investigated the transcription pattern of *var* genes by real-time polymerase chain reaction, the expression of VAR2CSA, protein by flow cytometry, and the CSA-binding ability of IEs collected at different stages of pregnancy using a static-based Petri dish assay.

Results. Through comparison with the profiles of isolates from nonpregnant hosts, we report several lines of evidence showing that parasites infecting women during pregnancy preferentially express VAR2CSA protein, and that selection for the capacity to adhere to CSA via VAR2CSA expression occurs early in pregnancy.

Conclusions. Our data suggest that the placental tropism of *P. falciparum* is already established in the first trimester of pregnancy, with consequent implications for the development of the pathology associated with placental malaria.

Malaria in pregnancy is an important cause of maternal anemia, stillbirth, and delivery of babies with low birth weight, the latter representing a major risk factor for infant mortality in Africa [1]. It is a well-established fact that pregnant women are more susceptible to infection with *Plasmodium falciparum* from the first trimester onward, with peaks of peripheral parasite density between 13 and 16 weeks of pregnancy [1], but the characteristics of these infections are poorly described. Relationships between parasitemia occurring in the first trimester and poor pregnancy outcomes, both in terms of birth weight and maternal anemia, have been reported [2, 3], highlighting the risk to which both the mother and fetus are exposed in early gestation. Paradoxically, current preventive measures for malaria during pregnancy do not cover this period, first because attendance rates at antenatal clinics by women in their first trimester are generally low with commensurately low rates of detection and treatment of infections, and second because, for reasons of safety, the currently recommended intermittent preventive treatment for malaria during pregnancy is implemented only after the first trimester. Potentially pathogenic infections arising in early pregnancy thus merit more detailed study.

The increased susceptibility to infection with *P. falciparum* during pregnancy of women who have previously acquired a degree of immunity is associated with sequestration within the placenta of infected erythrocytes (IEs) that express unique variant surface antigens (VSAs) mediating adherence to chondroitin

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sulfate A (CSA) (reviewed in [4]). *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1) molecules coded by *var* genes are expressed on the surface of the IEs and are the main VSAs that mediate adhesion of the IEs to various host receptors [5, 6]. Association between *var* gene expression and clinical presentation of malaria and/or outcome has been investigated in several studies that revealed their crucial role in malaria pathogenesis [7–10]. The transcription of *var* genes belonging to groups A and/or B has been associated with symptomatic and severe malaria [8, 10, 11], whereas *var* group C expression is associated with asymptomatic malaria [11]. The *Pf*EMP1 variant encoded by the *var2csa* gene was identified as the particular parasite ligand for CSA [12–15], and is associated with malaria in pregnancy [7, 9, 16]. Parasites isolated from placental blood selectively transcribe the *var2csa* gene, as do parasites selected for IE adhesion to CSA [7, 9, 17, 18]. High plasma levels of antibodies against VAR2CSA are associated with a lower frequency of the poor birth outcomes associated with *P. falciparum* infection during pregnancy [13, 19]. Furthermore, VAR2CSA-specific immunoglobulin G (IgG) can efficiently block IE adhesion to CSA [20, 21]. Given this knowledge, the VAR2CSA protein has become the major candidate antigen for the development of a vaccine designed to protect pregnant women from placental malaria.

Because there are still uncertainties about the onset of *var2csa*-specific transcription in pregnancy, we assessed the abundance of *var* gene transcripts in parasites collected from women at different times during pregnancy, as well as in patients with uncomplicated malaria and in children with cerebral malaria. Simultaneously, we investigated the expression of VAR2CSA on the surface of IEs and their binding properties using a standardized in vitro CSA-binding assay.

MATERIALS AND METHODS

Ethics Statement

The study was approved by the Comité Consultatif de Déontologie et d'Ethique of the Research Institute for Development (France) and the ethical committee of the Faculty of Health Science (University of Abomey-Calavi, Benin). All procedures complied with European and France national regulations. Animal immunization followed the Federation of Laboratory Animal Science Associations guidelines and was approved by the Comité d'éthique en matière d'expérimentation animale affiliated with the Université Paris Descartes.

Study Design, Malaria Patients, Sample Collection, and Storage

The study was conducted in Comé, a semirural area of southern Benin, at the Centre Hospitalier Universitaire Hubert Koutoucou Maga (CHU-HKM) and at Hôpital Mère-enfant de la Lagune (HOMEL) in Cotonou, Benin. The detailed description of the area has been reported elsewhere [2]. In brief, southern Benin is a high malaria transmission area with peaks during the 2 rainy seasons. *Plasmodium falciparum* is the predominant malaria-causing parasite, and the entomological inoculation rate ranges from 35 to 60 infective bites per person per year [22]. Pregnant women were enrolled during their first or second trimester of pregnancy (gestational age <24 weeks), and were followed until delivery. A *P. falciparum* rapid diagnostic test was performed on capillary blood, and 10-mL venous blood samples were collected at enrollment and during each antenatal or emergency visit. At delivery, peripheral and perfused placental blood samples were also collected. Ultrasound scans were performed with a portable ultrasound system (Titan Sonosite) to determine the exact gestational age. Two to 4 mL of venous blood were also collected from children <5 years at CHU-HKM and HOMEL presenting with cerebral malaria, defined according to World Health Organization criteria. All included children had a Blantyre coma score ≤2. Nonpregnant patients admitted to the health center with fever and a rapid diagnostic test positive for plasmodial infection and no signs of complicated malaria were also recruited.

Erythrocyte pellets (200 μL) were conserved either in Trizol reagent (Invitrogen) stored at −80°C or as spots (25 μL) on dried Whatman 3MM filter paper stored at room temperature. Pellets of 200 μL were cryopreserved and 300–700 μL was immediately cultured in vitro to trophozoite stage. In total, samples were collected from 50 parasitized pregnant women at enrollment (PW early); 41 parasitized pregnant women at delivery; 36 patients with uncomplicated malaria (UM), and 39 children with cerebral malaria (CM).

Parasite Culture and Selection

Primary field *P. falciparum* IEs obtained from the peripheral blood were maintained in vitro for no more than 48 hours before testing [23]. FCR3 and HB3 standard laboratory-adapted parasite cultures were grown in O+ erythrocytes without human serum and were selected following several panning steps on the choriocarcinoma cell line BeWo, as described by Haase [24].

Antibody Production and IgG Preparation

Specific anti-VAR2CSA IgG was produced in rabbits by DNA vaccination using DNA sequence encoding the full-length *var2csa* gene from the FCR3, as described [21]. In brief, animals were immunized 3 times with the *var2csa* sequence and cloned into a pVax1 vector backbone (Invitrogen) as described elsewhere [25]. Blood was collected at day 0 and 15 days after the last immunization, and total IgG was purified on a Hi-Trap Protein G HP column, according to the manufacturer's recommendations (GE Healthcare).

Flow Cytometry and Enzyme-Linked Immunosorbent Assays

Flow cytometry (FACSCalibur) was used to assess VAR2CSA expression on the surface of *P. falciparum* IEs isolated from patients using anti-VAR2CSA IgG as described [20]. The recognition of native VAR2CSA on late-stage *P. falciparum* laboratory lines (unselected FCR3 and FCR3-BeWo selected) by plasma from all groups was investigated as previously described [21].

Microtiter plates (Nunc 442 404) were coated with 0.5 μg/mL of each recombinant protein, DBL3X and DBL5ε domains of VAR2CSA [16], and AMA1. Plasma levels of specific IgG were measured in samples from pregnant women, children with CM, and UM patients, using enzyme-linked immunosorbent assay as described elsewhere [19, 26]. Values were converted into arbitrary units, as previously described [26].

Binding Assay

A static Petri dish assay was used to determine the capacity of IEs to bind to chondroitin sulfate proteoglycan (CSPG), as described previously [21]. In brief, late-stage IEs enriched by filtration on magnetic column (VarioMACS, Miltenyi), with a parasite density adjusted to 20%, were blocked in bovin serum albumin (BSA/RPMI) and allowed to bind to ligand (coated as spots in a 100 × 15-mm Falcon 351 029 Petri dish) for 15 minutes at room temperature (RT). Nonadherent cells were removed by an automated washing system. Spots were fixed with 1.5% glutaraldehyde in phosphate-buffered saline and stained with Giemsa, and adherent IEs were quantified by microscopy.

Genomic DNA and *msp* Genotyping

Genomic DNA was extracted from the filter spots as previously described by use of Chelex [27]. *Msp1* and *msp2* genes were amplified by nested polymerase chain reaction (PCR) using specific primers [28]. Multiplicity of infection was determined as the highest *msp1* or *msp2* allele number detected in each sample.

RNA Extraction and Complementary DNA Synthesis

Total RNAs were extracted from thawed samples stored in TRIzol reagent Invitrogen, as recommended by the manufacturer. RNAs were treated with DNase I (Invitrogen) and absence of genomic DNA (gDNA) in RNA samples was confirmed by no amplification after 40 cycles of real-time PCR with seryl-tRNA synthetase primers [12]. Reverse transcription of DNA-free RNA was performed using Thermoscript (Invitrogen) with random hexamer primers, as recommended by the manufacturer.

Quantification of *var* Gene Transcripts

Runs were performed on a Rotorgene thermal cycler system (Corbett Research) in 20 μL final volume, using 0.5 μL complementary DNA; 1× SYBR Green Mastermix (Bioline) and 1.25 μM of specific primer pairs for individual gene or *var* gene subtypes. Genomic DNA from the 3D7 parasite line was used as standard in the transcript analyses. Seryl-tRNA synthetase (primer pair p90) and fructose-bisphosphate aldolase (primer pair p61) were used as endogenous controls [12]. A universal primer pair targeting the conserved region of *var2csa* [7] and specific *var*-type primers previously designed were used as described elsewhere [8]. All primers were tested on 10-fold dilution of 3D7 gDNA, to determine primer amplification efficiencies (Supplementary Table 1). The melting curve analysis was done to ensure the amplification specificity. Nontemplate controls and the 3D7 gDNA (calibrator) were performed for validation on every run.

Statistic Analysis

Categorical variables were compared using the Fisher exact test, and continuous variables were compared by the Kruskal-Wallis test. Transcription of *var* gene was expressed as relative copy number and compared between all clinical groups using the Kruskal-Wallis test, or the Wilcoxon rank sum test when 2 groups were compared. Comparison of serological recognition of specific antigens by plasma from different groups of patients was done similarly. Data were plotted using Prism software (version 4; GraphPad) and Stata 12 software was used for statistical analyses. *P* values <.05 were considered to be statistically significant.

RESULTS

Clinical Characteristics of Participants

The median gestational age was 15.9 weeks (interquartile range [IQR], 13.7–17.0 weeks) at enrollment, and 39.4 weeks (IQR, 37.9–40.3 weeks) at delivery. Twenty-six of the pregnant women included were primigravidae. Other main characteristics of the study participants are presented in Table 1.

Prevalence of *var* Gene Transcription

Transcripts from *var* group A were detected in all 36 (100%) isolates from children with CM, 18 (82%) UM isolates, 19 (38%) PW isolates at early pregnancy, 31 (79%) peripheral isolates, and 19 (95%) placental isolates from pregnant women at delivery (Table 2). *Var* group B and C gene transcripts were detected in <35% of the early pregnancy isolates, but in more than 50% and 92% of the UM and CM isolates, respectively. Transcripts of *var2csa* were detected in all (100%) peripheral and placental isolates of pregnant women at delivery, in 90% of isolates at early pregnancy, 97% of CM isolates, and 86% of UM isolates.

Transcript Abundance of *var* Genes

The relative copy number defined for the distinct targets analyzed varied between isolates, covering at least a 6-log range with very low level observed in some samples. On average, the relative copy number of *var* genes belonging to group A was higher in children with CM compared to the UM and PW

Table 1. Clinical and Parasitological Characteristics of the Study Population

	Malaria Forms			
			Pregnant Women	
Characteristics	UM (n = 36)	CM (n = 39)	PW Early (n = 50)	PW Delivery (n = 41)
Sex (female/male)	17/19	17/22	50/0	41/0
Age, years median (IQR)	18 (11.7–34.2)	3 (2–4)	22 (19–28)	23 (20–30)
Median parasite density (IQR), parasites/μL	2500 (198–23 115)	104 000 (7000–420 000)	282 (126–656)	1265 (36–3228)
Hemoglobin. median (IQR), g/dL	11.7 (10.3–13.9)	5.7 (3.9–8.5)	10.1 (9.2–11.1)	10.8 (10.1–11.2)
MOI, median (IQR)	3 (2–4)	5 (3.2–6)	4 (3–5)	3.5 (2.2–4.7)

Abbreviations: CM, children with cerebral malaria; IQR, interquartile range; MOI, multiplicity of infection; PW, pregnant women; UM, patients with uncomplicated malaria.

groups (Figure 1) ($P < .0001$). However, at the individual level, only *var* A types were consistently the dominant transcripts detected in CM samples. No significant difference was observed in transcript abundance of groups B and C *var* among isolates from the different groups of patients. The *var2csa* gene copy number was significantly higher in isolates from pregnant women than in isolates from other groups, irrespective of their temporal or spatial origin ($P < .0001$). *Var2csa* was the dominant *var* transcript among all samples from pregnant women collected at delivery. This was not the case in 5 of 50 (10%) of the isolates collected early in pregnancy, in which the dominant *var* genes were of the B and C types. Of these 5 isolates, 4 were from pregnant women with gestational age (GA) less than the median value (7.4, 12.4, 13.0, and 13.3 weeks), and 1 from a pregnant woman at 20 weeks of GA. In isolates that transcribed *var2csa*, the transcript abundance of *var2csa* did not vary with GA ($GA < 13$; $13 \leq GA < 17$, and $17 \leq GA < 21$) and was similar to transcript levels at delivery (Figure 2*A*). At delivery, the level of *var2csa* transcription was similar in peripheral and placental isolates (Wilcoxon matched pair signed rank test, $P = .60$).

VAR2CSA Expression on the Surface of Infected Red Blood Cells From Patients With Distinct Clinical Forms of Malaria

A total of 62 of 91 PW, 22 of 36 UM, and 21 of 39 CM isolates were successfully matured in vitro. Higher reactivity of anti-VAR2CSA IgG was observed with IEs from pregnant women (median fluorescence intensity [MFI] ratio median, 2.98 [IQR, 1.6–4.6]) compared with IEs from CM (MFI ratio median, 1.24 [1.0–1.7]) and UM (MFI ratio median, 1.04 [1.0–1.1]) patients ($P < .0001$, Figure 2*B*). Significant labeling of IEs was observed in 53 PW isolates, but also in 4 UM and 3 CM isolates. The characteristics of the non-PW isolates that were labeled with anti-VAR2CSA are shown in Supplementary Table 2. No significant labeling was observed for the majority of UM and CM isolates despite the presence of *var2csa* transcripts in those samples. Although recognition of IEs from pregnant women was variable, this was not related to gestational age ($P = .30$) (Figure 2*C*). Of particular note, 10 of the 13 isolates collected before the 13th week of gestation consistently expressed VAR2CSA on the surface of IEs. Comparison of isolates with low and high reactivity segregated according to the median MFI value showed that those isolates with a lower *var2csa* gene copy number exhibited lower reactivity with anti-VAR2CSA IgG ($P = .02$), demonstrating that VAR2CSA expression on IE is directly linked to transcript abundance.

Adhesion Ability of *P. falciparum* IEs Collected From Pregnant Women

CSPG supported significant levels of adhesion of IEs while adhesion to BSA and adhesion of unselected strains to CSPG was generally <5 parasites/mm^2. The different isolates

Table 2. Prevalence of *var* Gene Types Transcribed in Parasites From Patients With Distinct Malaria Syndrome

	A1	A2	A3	B1	B2	C1	C2	BC1	BC2	Var1	Var2	Var3
UM (n = 36)	68.2	63.6	68.2	45.5	50.0	72.7	72.7	100	81.8	59.1	86.4	59.1
CM (n = 39)	97.2	100	86.1	91.7	97.2	88.9	88.9	100	94.4	86.1	97.2	97.2
PW Early (n = 50)	32.0	30.0	34.0	24.0	24.0	30.0	34.0	54.0	36.0	32.0	90.0	44.0
PW Per (n = 39)	71.8	71.8	74.4	62.2	82.1	71.8	79.5	82.1	87.2	71.8	100	74.4
PW Pla (n = 20)	90.0	95.0	80.0	80.0	95.0	95.0	95.0	100	95.0	90.0	100	95.0

Values indicate the proportion of parasites in which the transcript was detected in each group.

Abbreviations: CM, children with cerebral malaria; Per, peripheral blood at delivery; Pla, placental blood at delivery; PW, pregnant women; UM, patients with uncomplicated malaria.

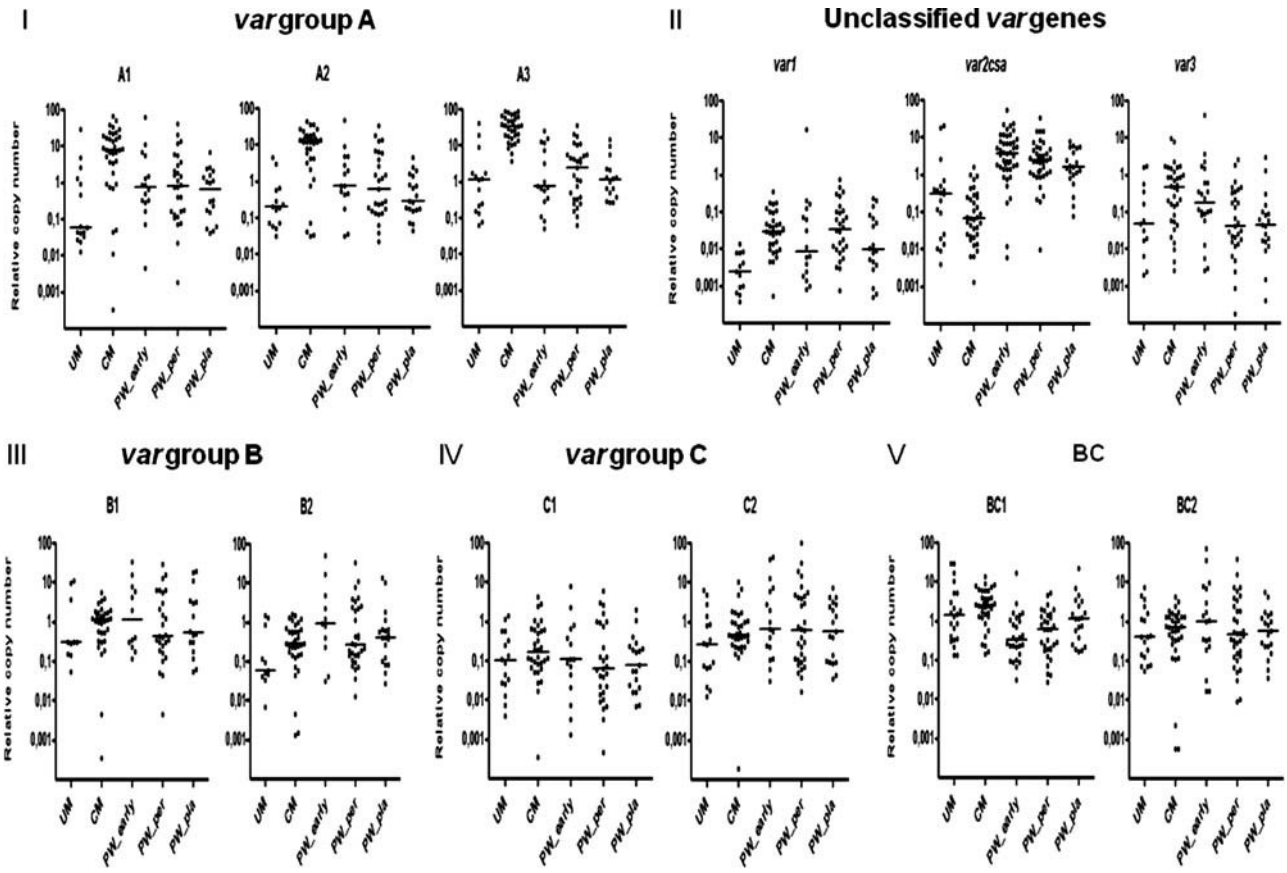

Figure 1. Transcription of *var* genes in parasites from patients with distinct clinical forms of malaria. Parasites were collected from pregnant women (PW), patients with uncomplicated malaria (UM), and children with cerebral malaria (CM). Transcript abundance is shown as log-transformed relative copy number. Parasites from pregnant women were collected at enrollment in early pregnancy (PW_early), and at delivery from peripheral blood (PW_per) and from placental blood (PW_pla). The real-time polymerase chain reaction conditions were as follows: 95°C for 1 min, followed by 40 cycles of 94°C for 30 s, 54°C for 40 s, and 68°C for 50 s. Samples with cycle threshold (Ct) values exceeding the linearity of dilution curves (Ct >35) were not quantified. The relative copy number was determined using the formula $C/E^{\Delta CT}$ where C is the number of *var* gene copies in the corresponding *var* group of the 3D7 genomic DNA [8], and ΔCT is the difference in Ct values between the sample and the corresponding *var* group plate calibrator [10, 18]. The target *var* gene copies were normalized by seryl-tRNA synthetase copy number for the amount of total DNA loaded in each reaction.

displayed variable adhesion capacities but that capacity did not differ according to the period of pregnancy from which the parasites were collected ($P = .49$, Figure 2*D*). Of particular interest, peripheral parasites collected before the 13th week of gestation bound CSPG equally as well as those collected late in pregnancy. The characteristics of early pregnancy isolates are shown in Table 3.

Naturally Acquired VAR2CSA-Specific IgG

Plasma from primigravid PW, children with CM, and UM patients recognized the surface of the CSA-adhering/VAR2CSA–expressing parasite strain (FCR3-BeWo) and the unselected FCR3 lines in the same manner (Figure 3*A*). However, a significantly higher level of recognition of the CSA-selected FCR3-BeWo IEs was found with plasma of multigravidae women ($P < .0001$). Irrespective to timing of pregnancy, plasma from pregnant women displayed markedly stronger recognition of VAR2CSA antigens compared with those of nonpregnant patients ($P < .0001$), with the highest levels by samples from multigravidae patients (Figure 3*B* and 3*C*). High levels of anti-AMA1 IgG were detected in samples from all participants, although there was a tendency toward an increase with age (Figure 3*D*).

DISCUSSION

Accumulated evidence suggests that differential expression of the various *var* types or subgroups may be involved in parasite virulence. To further explore this issue in the particular context of malaria during pregnancy, we collected and characterized *P. falciparum* isolates from groups of individuals presenting with different malaria syndromes in southern Benin. Although many distinct *var* gene transcripts were detectable

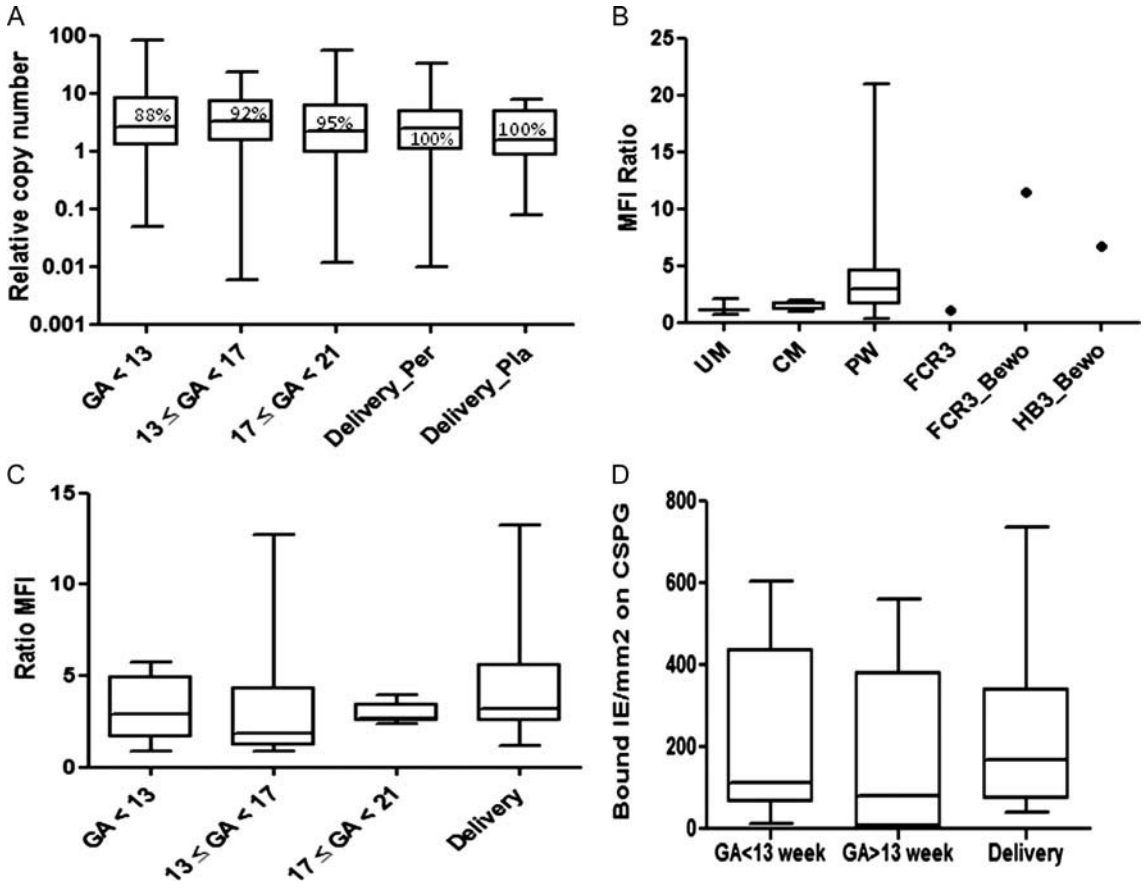

Figure 2. VAR2CSA expression and chondroitin sulfate proteoglycan (CSPG) binding ability of isolates from pregnant women (PW). *A*, Transcript abundance of *var2csa* in parasites collected from PW at different stages of pregnancy. Parasite donors at enrollment in early pregnancy were segregated based on gestational age as GA < 13, 13 ≤ <17, and 17 ≤ GA <21. The prevalence of parasites in which the transcript was detected is indicated within each box. *B*, Recognition level of native VAR2CSA on the surface of isolates from patients with uncomplicated malaria (UM), children with cerebral malaria (CM), and PW, as well as 3 laboratory strains (FCR3, FCR3-BeWo, and HB3-BeWo), using purified immunoglobulin G (IgG) induced against the full-length VAR2CSA from the FCR3. For flow analyses, 2×10^5 infected erythrocytes (IEs) were labeled with ethidium bromide, and sequentially exposed to rabbit IgG or human serum, and antirabbit or antihuman IgG-FITC (Invitrogen). The flow reactivity shown is defined as the ratio of median fluorescence intensity (MFI ratio: MFI with anti-VAR2CSA IgG/MFI with control IgG). VAR2CSA surface expression was considered positive with an MFI ratio >1.2. *C*, VAR2CSA recognition on the surface of IEs collected from peripheral blood of women, at different stages of pregnancy. *D*, Data from chondroitin sulfate A (CSA) binding assays were successfully obtained for 33 isolates, of which 8 were collected before 13 weeks (GA < 13) of pregnancy, 11 after 13 weeks (GA > 13) of pregnancy, and 14 at delivery. Boxes delimit medians and interquartile ranges. Bars indicate the extreme values.

in all *P. falciparum* infections, only the analyses of the abundance of each transcript identified revealed clear differences among groups. Higher transcript levels of group A *var* types were found in isolates from children with CM, consistent with the findings of several previous studies [8, 29, 30]. The amount of group B *var* gene transcripts did not differ between groups contrasting with observations that reported high rates of their transcription in children with severe malaria [8, 10]. This suggests that CM parasites probably display particular patterns of *var* gene transcription [11, 31]. As expected, no major difference was found in transcript levels of group C *var* members, suggesting that members of this group do not influence the differing clinical outcomes of infection presented by the individuals studied here.

Our data confirm previously published findings that parasites from pregnant women highly transcribe the *var2csa* gene, regardless of their provenance from peripheral or placental blood at delivery [7, 9, 17, 18, 27]. Our fundamental objective, however, was to characterize parasites infecting women early in pregnancy. In this context, the fact that we used ultrasound to generate accurate estimates of gestational age not only distinguishes our study from others, but provides an ideal window through which to view well-defined early-onset infections. We found that parasites collected from pregnant women preferentially transcribed *var2csa* at high levels irrespective of gestational age. Of particular interest, the majority of parasites isolated in the first trimester (GA <13 weeks) also transcribed *var2csa* at high levels. We also demonstrated the presence of

Table 3. Binding Ability of Isolates Collected in the Peripheral Blood From Women Before 13 Weeks of Gestation

Isolate	Bound IE/mm^2 on BSA	Bound IE/mm^2 on CSPG	Parasites/μL in Blood	ParityStatus	Gestational Age
Earl-O32	2	82	181	1	7
Earl-O36	2.5	12	1058	1	10
Earl-O41	4	143	200	5	10
Earl-O21	3	70	9435	1	12
Earl-O25	2	61	206	2	12
Earl-O31	1.5	499	2620	1	12
Earl-O37	0.5	250	350	3	12
Earl-O73	2.5	605	3674	5	12

Binding of 8 *Plasmodium falciparum* IEs collected from pregnant women is shown as bound IEs/mm^2 to chondroitin sulfate proteoglycan. Control binding to bovin serum albumin is shown for each parasite. Parasite density, parity status of the woman, and the gestational age at the time of parasite collection are indicated.

Abbreviations: BSA, ; CSPG, ; IE, infected erythrocytes.

VAR2CSA on the surface of the majority of isolates from pregnant women. These observations corroborate the *var2csa* transcription data, and further emphasize the particular phenotypic characteristics of the parasites that infect pregnant women. The dominantly transcribed *var* gene during a given cycle is expected to be translated into a *Pf*EMP1 protein mediating a specific phenotype. The transcriptional profile of UM isolates did not show such a preference for a type or subfamily of transcripts, so we may expect that different *Pf*EMP1 variants are coexpressed in these less homogeneous parasite

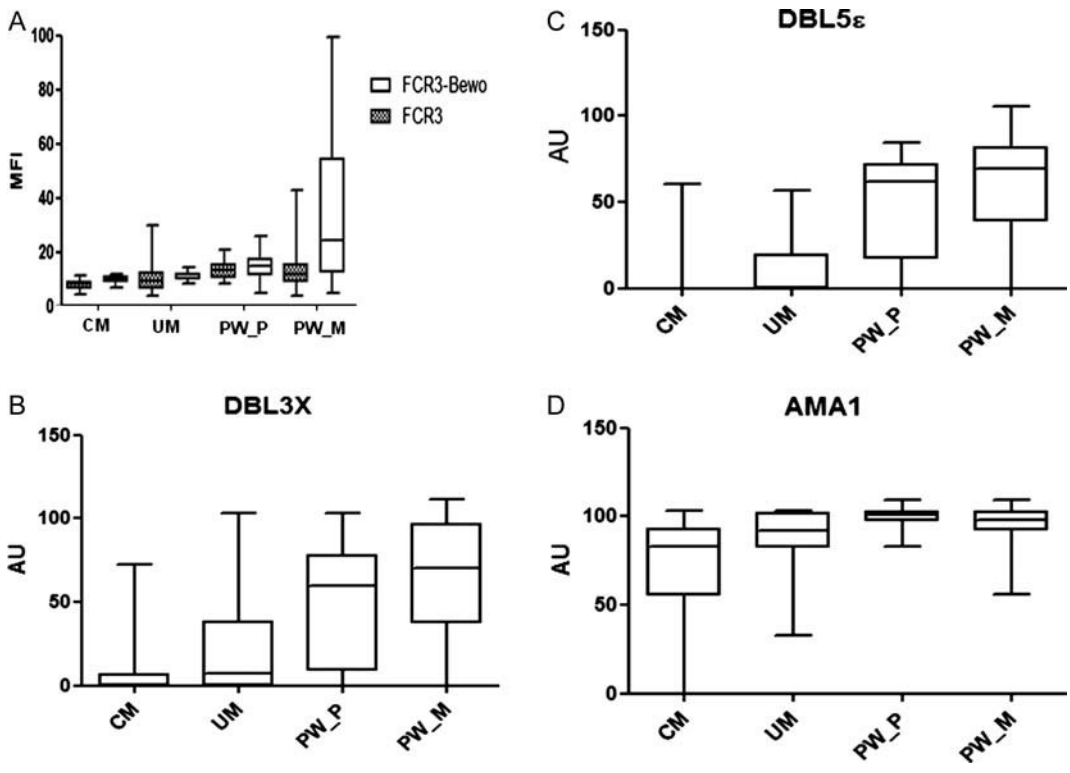

Figure 3. Plasma recognition of nonpregnancy and pregnancy-specific antigens. *A*, Plasma level of antibodies against unselected (FCR3, *dotted filled histograms*) and selected VAR2CSA-expressing (FCR3-BeWo, *white filled histograms*) parasite strains in samples from patients with uncomplicated malaria (UM), children with cerebral malaria (CM), and pregnant women (primigravidae: PW_P, and multigravidae: PW_M). The flow reactivity shown is defined as the median fluorescence intensity (MFI). Plasma levels of anti-VAR2CSA (DBL3X, DBL5ε) and anti-AMA1 immunoglobulin G (IgG) measured by enzyme-linked immunosorbent assay are shown in panels (*B*), (*C*), and (*D*), respectively. Data are expressed as arbitrary units (AUs). Boxes delimit medians and interquartile ranges. Bars indicate the extreme values.

populations. Indeed, a low level of IE reactivity of VAR2CSA-specific antibodies was observed in 7 isolates from nonpregnant women. This was an unexpected finding, as VAR2CSA expression is assumed to be restricted to placenta-sequestering parasites [32]. These results are nevertheless consistent with those of Beeson et al [33] and Oleinikov et al [34], indicating that surface expression of VAR2CSA can occasionally occur during infections in nonpregnant hosts, and the potential immunological impact of such parasites merits more detailed investigation.

Previous studies have shown that antibodies specific for VSA expressed by infecting parasites can be identified in plasma samples collected from parasite donors [35, 36]. Our data provide confirmation of the presence of VAR2CSA-specific antibodies predominantly in the plasma of pregnant women. That a limited number of nonpregnant women possess anti-VAR2CSA IgG is consistent with the detection of the protein on IE from such individuals. Our results thus support our own previous observations concerning the transcription and surface expression of VAR2CSA in *P. falciparum* IEs isolated from pregnant women at delivery [7, 16]. More importantly, they show for the first time that the overwhelming majority of parasites causing infections early in pregnancy already exhibit VAR2CSA-expressing and CSA-adhering phenotypes. Although previous data showed that anti-VAR2CSA antibodies are acquired early in pregnancy (from the second trimester), strongly suggesting infection with VAR2CSA-expressing parasites [19, 37], current finding on parasites collected as early as 7 weeks is to some extent unexpected as the intervillous spaces of the placenta are purportedly not vascularized, and therefore not accessible to maternal blood before the 12th week of gestation [38], prior to which the fetus develops in a hypoxic medium [39]. Without access to intervillous spaces, IEs should, theoretically at least, not encounter and adhere to the CSA expressed there. However, current knowledge concerning the onset of placental vascularization may be insufficiently precise. Using transvaginal color Doppler, intervillous blood flow from the sixth week of healthy pregnancies has been reported [40, 41], suggesting that placental irrigation may occur much earlier than previously thought. It is also possible that the fetus-derived extravillous trophoblasts that invade maternal uterine spiral arteries may themselves express CSA, since they share the same origin as CSA-expressing syncytiotrophoblasts. Such cells could plausibly come into contact with IEs during the first trimester to provide them with an initial niche for sequestration. Although the mechanism involved remains to be elucidated, that women at early gestational ages may harbor potentially pathogenic VAR2CSA-expressing *P. falciparum* parasites emphasizes the urgent need to address the issue of malaria prevention early in pregnancy. This urgent need is supported by recent field studies that showed relationships between infection occurring in the first trimester and poor pregnancy outcomes, both in terms of birth weight and maternal anemia [2, 3].

In summary, our data show that parasites infecting women during pregnancy preferentially express VAR2CSA, the parasite ligand for the placental receptor CSA, and that selection in vivo for those particular parasites occurs much earlier in pregnancy than has been appreciated to date. Although current prevention measures for malaria in pregnancy promote the use of insecticide-treated nets from as early as possible in pregnancy, and the institution of intermittent preventive treatment during pregnancy is recommended after quickening, these measures do not ensure optimum efficacy to address this issue, a situation exacerbated by the fact that placental malaria is predominantly asymptomatic, and therefore goes undetected and untreated. A syndrome-specific vaccine that would ensure effective protection against placental malaria from conception through to delivery appears an even more desirable goal.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Article II: Dynamics of Plasmodium falciparum infected erythrocytes adhesion phenotype during pregnancy associated malaria

Doritchamou J, Sossou-tchatcha S, Cottrell G, Moussiliou A, Houngbemin HC, Massougbodji A, Deloron P, Tuikue Ndam N.

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Dans la précédente étude, il est apparu que bien que certains isolats du premier trimestre exprimaient clairement un phénotype placentaire typique. Cette propriété était variable d'un isolat à l'autre et certains adhéraient soit très faiblement, soit pas du tout au CSPG. Ces observations ont amené à une nécessité d'étendre ce travail à d'autres récepteurs connus afin de mieux analyser les propriétés d'adhérence des parasites qui infectent les femmes tout au long de la grossesse. Plusieurs études ont montré que les isolats parasitaires de *P. falciparum* provenant de femmes enceintes adhèrent préférentiellement à la CSA, faisant de cette molécule, le principal sinon le seul récepteur impliqué dans la séquestration placentaire des EI (9,62,70,75,141,155,156,182) tandis que des parasites isolés d'individus non enceintes adhèrent fortement au CD36 et/ou ICAM-1 selon la forme clinique. Par ailleurs, il a été montré que ces deux récepteurs sont fortement exprimés dans le placenta. ICAM-1 a été localisé sur les syncytiotrophoblastes, suggérant ainsi une possible implication de ces récepteurs dans la séquestration placentaire des EI (183). La fonction et le niveau d'implication de ces molécules dans les propriétés d'adhérence des EI isolés des femmes enceintes sont encore mal élucidés. Notre étude avait pour but de caractériser les propriétés d'adhérence *ex vivo* des isolats frais collectés chez les femmes enceintes à différents moments de la grossesse, sur les récepteurs CSPG, CD36 et ICAM-1.

La transcription des gènes *var* a pu être quantifiée par qPCR sur 100 isolats provenant de femmes incluses. Tous les isolats ont été mis en culture pour atteindre les formes matures du parasite nécessaire à une expression optimale des protéines parasitaires. Le niveau d'expression en surface de VAR2CSA des EI et l'analyse du pouvoir d'adhérence des isolats sur les trois récepteurs ont été évalués sur 54 isolats pour lesquels la maturation des parasites *in vitro* a été possible.

Les résultats montrent une diversité de phénotype d'adhérence aux récepteurs étudiés, bien que l'adhérence au CSPG reste le phénotype majoritaire commun aux isolats de femmes

enceintes quelque soit le moment d'infection au cours de la grossesse. Toutefois, une plus grande diversité a été observée en début de grossesse, celle-ci se resserrant progressivement avec l'âge gestationnel en faveur du phénotype d'adhérence au CSPG. Cela souligne le risque de plus en plus élevé d'infection des femmes par des parasites à tropisme placentaire avec le développement et l'irrigation croissante du placenta. Ceci soutient les précédents travaux qui ont montrés que la capacité des anticorps à inhiber l'adhérence des EI à la CSA était associée à l'âge gestationnel (159). Il est également apparu que les parasites infectant les femmes enceintes primigestes adhèrent plus au CSPG comparativement à ceux isolés des femmes multigestes. Ces résultats confortent le risque élevé d'infection des femmes primigestes par des parasites pouvant adhérer à la CSA en l'absence d'une immunité protectrice qui s'acquière au cours des grossesses successives (6,158,184). Inversement, la prévalence des parasites adhérant à des récepteurs autres que le CSPG parait plus importante chez les femmes multigestes, chez qui l'immunité contre les parasites ayant un tropisme placentaire est mieux développée.

Ces données démontrent une dynamique des phénotypes d'adhérence des parasites chez femmes enceintes, celle-ci étant modulée d'une part par la physiologie de la grossesse (immuno-modulation et accessibilité du placenta avec l'irrigation croissante), et d'autre part par l'environnement immunitaire. Il est mis en lumière l'intérêt de caractériser les variants de VAR2CSA qui expriment une forte capacité d'adhésion à la CSA. C'est un aspect essentiel à considérer dans l'effort actuel de développement d'un vaccin à base de VAR2CSA.

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PLOS ONE

Dynamics in the Cytoadherence Phenotypes of *Plasmodium falciparum* Infected Erythrocytes Isolated during Pregnancy

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Abstract

Pregnant women become susceptible to malaria infection despite their acquired immunity to this disease from childhood. The placental sequestration of *Plasmodium falciparum* infected erythrocytes (IE) is the major feature of malaria during pregnancy, due to ability of these parasites to bind chondroitin sulfate A (CSA) in the placenta through the VAR2CSA protein that parasites express on the surface of IE. We collected parasites at different times of pregnancy and investigated the adhesion pattern of freshly collected isolates on the three well described host receptors (CSPG, CD36 and ICAM-1). *Var* genes transcription profile and VAR2CSA surface-expression were assessed in these isolates. Although adhesion of IE to CD36 and ICAM-1 was observed in some isolates, CSA-adhesion was the predominant binding feature in all isolates analyzed. Co-existence in the peripheral blood of several adhesion phenotypes in early pregnancy isolates was observed, a diversity that gradually tightens with gestational age in favour of the CSA-adhesion phenotype. Infections occurring in primigravidae were often by parasites that adhered more to CSA than those from multigravidae. Data from this study further emphasize the specificity of CSA adhesion and VAR2CSA expression by parasites responsible for pregnancy malaria, while drawing attention to the phenotypic complexity of infections occurring early in pregnancy as well as in multigravidae.

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Introduction

Despite the substantial protective anti-malarial immunity gradually acquired during childhood in residents of areas with high malaria transmission, during their first pregnancy women are more at risk of infection by *Plasmodium falciparum* compared to non-pregnant adults [1]. The sequestration of *P. falciparum*-infected erythrocytes (IE) in the placenta is the key characteristic of pregnancy-associated malaria (PAM), and can be associated with intense inflammatory activity. The latter is more common in women during their first pregnancy. The pregnancy-specific aspect has been attributed to parasites expressing particular variant surface antigens [2,3]. Severe maternal anemia and delivery of babies with low birth weight are the major consequences associated with the accumulation of IE in the placenta [4]. The best-characterized adhesion ligands expressed on the surface of IE are members of the highly polymorphic *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) family, encoded by the *var* gene family [5]. *Var* genes can be classified into 5 majors groups (A to E) based on the sequence polymorphism observed both in the non-coding upstream region and also in the coding sequence [2,6–8]. A particular PfEMP1, named VAR2CSA, is now recognized as the main parasite ligand mediating IE binding to placental tissue [2,9,10].

Numerous characteristics of VAR2CSA make it the major candidate for development of a vaccine to prevent PAM, characteristics that have been described in multiple studies [2,9,11–19]. However, data concerning the adhesion patterns of parasite isolates collected throughout pregnancy, and the kind of interactions that can characterize isolates present at different times of pregnancy, remain fully to be generated. Although it has been suggested that other molecules (hyaluronic acid and non-immune globulins) may participate in the adhesion of IE in the placenta [20–22], several lines of evidence indicate that CSA is the most important receptor involved [2,3,17,23–28]. Endothelial receptors, such as CD36 and ICAM-1, commonly support the adhesion of field isolates [26] from non-pregnant patients [29–31]. However, it has been shown that these two receptors are highly expressed in the placenta and ICAM-1 has been localized on syncytiotrophoblasts, suggesting a possible role in the placental sequestration of IE [32]. Other studies have nevertheless reported that placental isolates do not bind to CD36 [20,22] and ICAM-1

[25]. Thus, the function and the level of involvement of these molecules in the binding ability of IE collected from cases of PAM are still not well explored. In this study, we sought to characterize the binding properties *ex vivo* of field isolates collected from pregnant women at different time-points of pregnancy using three receptors expressed in the placenta that are known to support IE binding (CSPG, CD36 and ICAM-1). In addition, we investigated whether other pregnancy-related factors influence the parasite adhesion properties and whether infection by parasites with a particular adhesion pattern could be associated with poor pregnancy outcomes.

Material and Methods

Study design, collection and handling of blood samples

Written informed consent was given by all women participating in this study. The study was approved by the ethics committee of the Faculty of Health Science (University of Abomey-Calavi) in Benin. The study was conducted at the Suru Léré maternity clinic, Cotonou, Benin. All women were tested for *P. falciparum* infection using a rapid diagnostic test (Parascreen, Zephyr Biomedicals Goa, India), and those with a positive result were included. *P. falciparum* IE were obtained from 123 pregnant women attending antenatal visit and 9 women admitted for delivery. Venous blood was collected in vacutainers with citrate phosphate dextrose adenine anticoagulant. Thick and thin blood films were prepared from blood samples to confirm *P. falciparum* infection. Hemoglobin values of women and the birth weight of their offspring were collected for all women included at delivery. Detailed characteristics of the study site have been previously described [33].

Ring stage IE were allowed to mature *in vitro* to trophozoite-stage, as described [34]. Briefly, isolates were grown in RPMI 1640 supplemented with Hepes and L-glutamine (Lonza Biowhittaker), 0.3 g/L l-glutamine, 0.05 g/L gentamicin, 5 g/L albumax. Cultures were grown for no more than 48 h before testing. Ring stage parasites were also conserved in 10 volumes of TRIzol reagent (Invitrogen) and stored at -80°C until RNA extraction.

Flow cytometry and binding assays

VAR2CSA expression on the surface of *P. falciparum* IE was assessed by flow cytometry using specific anti-VAR2CSA IgG as previously described [35]. Briefly, 2×10^5 late-stage IE enriched by filtration on a magnetic column (VarioMACS, Miltenyi) were labelled with ethidium bromide, and sequentially exposed to anti-VAR2CSA rabbit IgG (final concentration 10 µg/ml), and to FITC-conjugated anti-rabbit IgG (1.5 mg/ml, Invitrogen). The anti-VAR2CSA rabbit IgG were purified from the plasma of rabbits previously immunized with the extracellular full-length protein from FCR3 strain [33]. A FACSCalibur flow-cytometer (BD Biosciences) was used to acquire the data, and the median fluorescence intensity (MFI) was determined. VAR2CSA surface expression was considered positive with an MFI ratio (MFI with IgG from rabbits immunized with VAR2CSA/MFI with IgG from rabbits before immunization) >1.2, as previously described [35].

A static assay that measures the adhesion to purified, immobilized receptors was used to assess the binding patterns of isolates, as described [36]. Briefly, 5 µg/ml of CSPG-Decorin (Sigma) or 10 µg/ml of ICAM-1 (R&D Systems) or CD36 (R&D Systems) or bovine serum albumin (Sigma) were diluted in PBS, and coated as spots in a 100×15 mm Petri dish (Falcon 351029). Late-stage IE enriched on a magnetic column (VarioMACS, Miltenyi), with a parasite density adjusted to 20% in 1×10^5 cells were blocked in BSA/RPMI for 30 minutes at room temperature (RT), and allowed to bind to coated receptors for 15 minutes at RT. Unbound cells were removed by an automated washing system. Bound IE were fixed with 1.5% glutaraldehyde in PBS, stained with Giemsa, and quantified by microscopy, as the number of IE bound per mm^2. Each sample was performed in duplicate. Based on the binding level of IE observed on BSA spots (data not shown), a threshold of significant adhesion was determined as the mean +3 standard deviations and was set as binding $\geq$35 IE/mm^2.

RNA extraction, cDNA synthesis and quantification of var gene transcripts

Thawed samples stored in TRIzol reagent were used to extract the total RNA, as recommended by the manufacturer. The dried pellet was resuspended with 10 µl of DEPC-water. RNA samples were treated with DNase I (Invitrogen) for 30 min at RT. The absence of gDNA in RNA samples was confirmed by no parasite DNA amplification after 40 cycles of real-time PCR performed with *seryl-tRNA synthetase P. falciparum*-specific primers, using a Rotorgene 6000 thermal cycler system (Corbett Research). Reverse transcription of DNA-free RNA was performed using Thermoscript (Invitrogen) with random hexamer primers in a total volume of 20 µl, as recommended by the manufacturer.

Var gene transcripts abundance was quantified by qPCR, as described [36]. Briefly, runs were performed (95°C for 1 min, followed by 40 cycles of 94°C for 30 s, 54°C for 40 s, and 68°C for 50 s) in a final volume of 20 µl, using 0.5 µl cDNA; 1×SYBR Green Mastermix (Bioline) and 1.25 µM of specific primer pairs for individual gene or *var* gene subtypes. Primer pairs targeting the conserved region of *var2csa* [9] and previously designed specific *var*-type primers (A1, B1, B2, C1, C2, *var1*, and *var3*) were used, as described [37]. *Seryl-tRNA synthetase* (primer pair p90) and *fructose-bisphosphate aldolase* (primer pair p61) were used as endogenous controls [2]. Non-template controls and the 3D7 gDNA, used as calibrator, were performed for validation on every run. The melting curve analysis was done to ensure the amplification specificity. Samples with Cycle Threshold (CT) values exceeding 35 were not quantified. The relative copy number of *var* genes transcripts was determined, as described [36].

Statistical analysis

Statistical analysis was performed using STATA software version 11 (Stata corporation, College Station, Texas, United States) and data were plotted using Prism software (version 5, Graph-Pad). Transcripts with abundance values greater than 5% of the total *var* genes analyzed were listed. Continuous variables were compared by the Mann-Whitney and Kruskall-Wallis tests. The Wilcoxon matched pairs test was used to compare matched variables. A linear regression model was used to analyze the binding level of parasites to each host receptor according to the parity of women, the timing of pregnancy and surface expression of VAR2CSA. The same analysis was performed on the data defined as positive and negative binding to each receptor using a logistic regression model. This latter model was performed, in addition to linear model, to describe and predict the binding pattern of isolates that infect women throughout pregnancy in relation with the parity status of these women.

Results

Transcription profile of var genes by isolates collected from pregnant women

Parasites were obtained from 132 pregnant women with a *P. falciparum* infection, as confirmed by microscopical examination. The clinical characteristics of these women are presented in Table 1. Analysis of *var* genes transcripts diversity was performed

Table 1. Clinical characteristics of the women and their offspring birth weight.

N = 132	Mean	Median	IQR
Parasitemia (/µl)	71,781	18,207	3,927–59,719
Age (years)	26.3	27	21–30
Parity	2.9	2	1–4
Gestational age (weeks)	22	23	12–28
Hemoglobin (g/dl)	9.6	9.7	8.8–10.6
Birth weight of the offspring (g)	3017	3000	2750–3350

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on 100 cDNA successfully synthesized. Although transcripts of several *var* genes were detected in most of the isolates, transcript of *var2csa* was detected in 99 out of the 100 tested cDNA. Isolates highly transcribed *var2csa* compared to other *var* genes ($P<0.0001$, Figure 1). The median copy number of *var2csa* detected among these isolates was 6.8 (IQR, 1.8–19.0) whereas other *var* genes coverage by specific primers targeting A1, B1, B2, C1, C2, *var1* and *var3* showed a median copy number <0.2. Moreover, *var1* was exclusively transcribed by one isolate (OPT173), and transcripts of *var2csa* were exclusively detected in eight isolates.

Adhesion phenotype of field isolates from pregnant women

To limit changes in the structure of parasite populations in the isolates studied, the culture time required *in vitro* to obtain mature stages used in the binding phenotyping was limited to 48h. Isolates with very low parasite density that required longer cultivation time to yield sufficient IE were not retained for analyses. The ability of parasites isolated from pregnant women to bind to host receptors (CSPG, CD36, and ICAM-1) was assessed on 54 successfully-matured isolates (50 samples collected during pregnancy and 4 samples obtained at delivery). Among these women, 13 were primigravidae and 41 were multigravidae. The levels of binding to each receptor (CSPG, CD36, and ICAM-1) are shown in Table 2. Distinct binding ability was observed among the parasite isolates. Although binding intensity also differed according to receptors, most of the tested isolates showed adhesion to at least one of the receptors (Table 2). Five isolates were not tested on CD36 and ICAM-1 due to limited amounts of IE. However, significant adhesion to CSPG was observed on 32 (59.2%) isolates, whereas 13 (26.5%) and 2 (4.1%) isolates showed substantial levels of binding to CD36 and ICAM-1, respectively (Figure 2A). Overall, isolates bound at significantly higher levels to CSPG (median = 81.5, 14.7–320.5) than to CD36 (8.0, 0–39.5) ($P=0.001$) and to ICAM-1 (0, 0.0–5.5) ($P<0.0001$). Furthermore, the binding intensity to ICAM-1 was also lower than to CD36 ($P=0.0004$), suggesting that affinity to this receptor may of less importance to isolates from pregnant women.

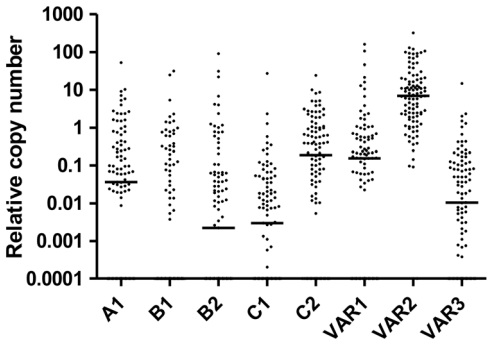

Figure1. *Var* genes transcription profile of isolates collected from pregnant women. Transcription level of *var* genes were shown as relative copy number. Bars indicate the median of distribution.
doi:10.1371/journal.pone.0098577.g001

Relationship between VAR2CSA expression and the CSA-binding phenotype

Var2csa is the only gene among the *var* genes examined in this study that was detected in all of the 54 successfully-matured isolates. To further refine the analysis we focus on the dominant transcripts detected. Only *var* genes with transcript abundances greater than 5% of the total *var* gene transcription [38], are reported in Table 2 and the most prevalent transcript indicates the dominant *var* gene of each isolate. Transcript abundance of *var2csa* above the threshold (5%) was observed in 48 out of 54 isolates whereas transcripts of *var1* and group ABC *var* genes were observed in 22/54 and in 25/54 (13/54 for *var* group A; 9/54 for *var* group B and 17/54 for *var* group C) of isolates, respectively (Table 2). *Var2csa* was exclusively or dominantly transcribed by 36 isolates. *Var1* was dominantly transcribed by 10 isolates, while *var* genes from groups ABC were dominantly transcribed by 6 isolates. The distribution of the transcription levels of the three categories of *var* genes (*var1*, *var2csa* and *var* ABC) was different in the two groups of parasite phenotypes (Fisher Exact Test; $P<0.0001$). Transcripts of *var1* was the predominant transcript detected (46%) among isolates with a CD36-binding phenotype (Table 3) while *var2csa* was (87%) in isolates binding to CSPG.

Transcription data and that of VAR2CSA expression on the surface of IE were further analyzed in relation to the adhesion properties of the isolates (Table 4). Among the 32 isolates that showed significant binding to CSPG, significant labeling of the IE surface by anti-VAR2CSA antibodies was observed while only one isolate (OPT091) was recognized among isolates which did not show a CSPG binding phenotype ($P=0.005$). Although all these CSA-adhering isolates transcribed *var2csa*, only 28 (87.5%) transcribed it as the dominant *var*. Among the 22 isolates which did not significantly bind to CSPG, *var2csa* was dominantly transcribed in 10 (45.4%), all of which were labeled by VAR2CSA antibodies. In five isolates a positive surface labeling was observed despite the fact *var2csa* was not the predominant transcript or just hardly detected.

Adhesion phenotype of parasites is associated with parity and gestational age of women

Isolates from primigravidae bound on average to a higher level to CSPG than those from multigravidae ($P=0.05$). Conversely,

Table 2. *Var* genes transcription profile and adhesion phenotype of 54 isolates collected from pregnant women in Cotonou, Benin.

Isolates	Dominant *var* gene(s) transcribed	Transcripts relative abundance (% of total *var*)	MOI	VAR2CSA surface detection	CSPG	CD36	ICAM-1
OPT079	*var2csa; upsC*	92; 6	2	1.2	41	nd	nd
OPT081	*upsB; upsA; upsC*	46; 31; 11	5	0	12	917	0
OPT091	*upsB; var1; upsA*	58; 26; 8	1	1.4	13	nd	nd
OPT101	*var2csa; upsC*	61; 39	4	1.2	28	nd	nd
OPT105	*var2csa*	99	4	2.7	255	nd	nd
OPT106	*var2csa; upsC; upsA*	71; 12; 10	4	1.2	148	nd	nd
OPT107	*var1; upsB; upsC; var2csa*	39; 29; 24; 7	3	1	8	903	0
OPT109	*var2csa*	99	5	2.3	275	21	17
OPT110	*var2csa*	98	6	1.4	261	13	0
OPT114	*var2csa*	98	2	2.4	0	5	0
OPT115	*var1; upsC; var2csa,*	49; 32; 17	1	1	7	111	64
OPT116	*var2csa; var3*	77; 23	1	2.4	15	39	0
OPT118	*var2csa*	99	4	3.6	250	0	0
OPT119	*var2csa; upsC; upsA*	50; 43; 6	2	3.3	10	19	12
OPT120	*var2csa; var1*	86; 9	3	1.5	14	414	6
OPT124	*var2csa*	94	1	3	87	65	0
OPT127	*var2csa; var1*	92; 5	1	3.4	446	14	0
OPT130	*var1; upsB; upsA*	70; 21; 7	4	4.1	746	6	9
OPT133	*upsC; var1; upsA; var2csa*	52; 17; 16; 8	1	0.9	7	175	32
OPT135	*var1; upsB; upsA; upsC; var2csa*	29; 24; 21; 18; 7	1	1	18	483	14
OPT137	*var2csa*	99	1	1.6	37	0	0
OPT139	*var2csa; upsB*	85; 6	3	1.5	6	32	0
OPT140	*var2csa*	98	4	1.6	253	8	0
OPT141	*var2csa*	99	3	3.7	492	6	0
OPT144	*var2csa*	97	3	3	414	8	0
OPT145	*upsC; var1; var2csa; upsA; var3*	29; 25; 19; 16; 6	2	1	10	28	0
OPT148	*var2csa; var1; upsC*	81; 7; 7	4	2	31	22	0
OPT151	*var2csa; upsC*	91; 7	4	3	715	0	0
OPT154	*var2csa; var1*	93; 5	2	3.4	632	0	0
OPT158	*var2csa; var1*	91; 7	2	1.9	108	0	0
OPT161	*var2csa*	98	4	2.5	401	0	0
OPT165	*var2csa; var1; upsB, upsC*	35; 31; 23; 8	2	0.8	5	0	9
OPT166	*var2csa*	97	4	1.6	41	0	0
OPT169	*var2ca*	98	1	6.7	263	0	0
OPT173	*var1*	100	1	0.3	0	91	0

Table 2. Cont.

Isolates	Dominant *var* gene(s) transcribed	Transcripts relative abundance (% of total *var*)	MOI	VAR2CSA surface detection	CSPG	CD36	ICAM-1
OPT175	*upsC; var2csa*	75; 20	3	0.8	27	65	6
OPT178	*var2csa*	98	2	2.6	54	0	14
OPT180	*var2csa; upsC*	93; 5	7	4	314	0	0
OPT184	*upsA; upsB*	57; 36	1	1.2	26	8	0
OPT220	*var2csa*	100	1	2.4	277	11	2
OPT225	*var2csa; uspA*	71; 28	3	1.9	136	29	1
OPT246	*var2csa; var1; upsA*	65; 22; 10	5	3.2	600	14	1
OPT248	*var2csa; uspC*	81; 18	3	2.9	126	40	5
OPT252	*var1; var2csa*	58; 40	4	1.8	340	107	4
OPT262	*var2csa; var1*	75; 18	5	3.8	509	5	1
OPT266	*var2csa; var1*	77; 20	3	1	183	7	4
OPT267	*var1; var2csa; upsA, upsC*	69; 13; 9; 6	3	4.3	354	13	4
OPT270	*var1; var2csa; upsB*	47; 43; 5	4	0.6	32	4	46
OPT272	*var1; var2csa; upsA*	52; 39; 5	5	0.8	15	44	23
PAM04	*var2csa*	96	1	2	28	0	0
PAM05	*var1*	94	2	2.5	76	0	0
PAM06	*var2csa*	99	1	7.6	0	5	0
PAM07	*var2csa*	97	1	3.1	1256	0	0
PAM08	*var2csa*	99	7	2.9	964	0	0

Transcription data are presented as the relative abundance of the total *var* studied. Only transcripts whose levels were greater than 5% of all transcripts detected are listed. Binding data corresponding to each receptor are expressed as the number of bound infected erythrocytes per mm^2 (IE/mm^2).

doi:10.1371/journal.pone.0098577.t002

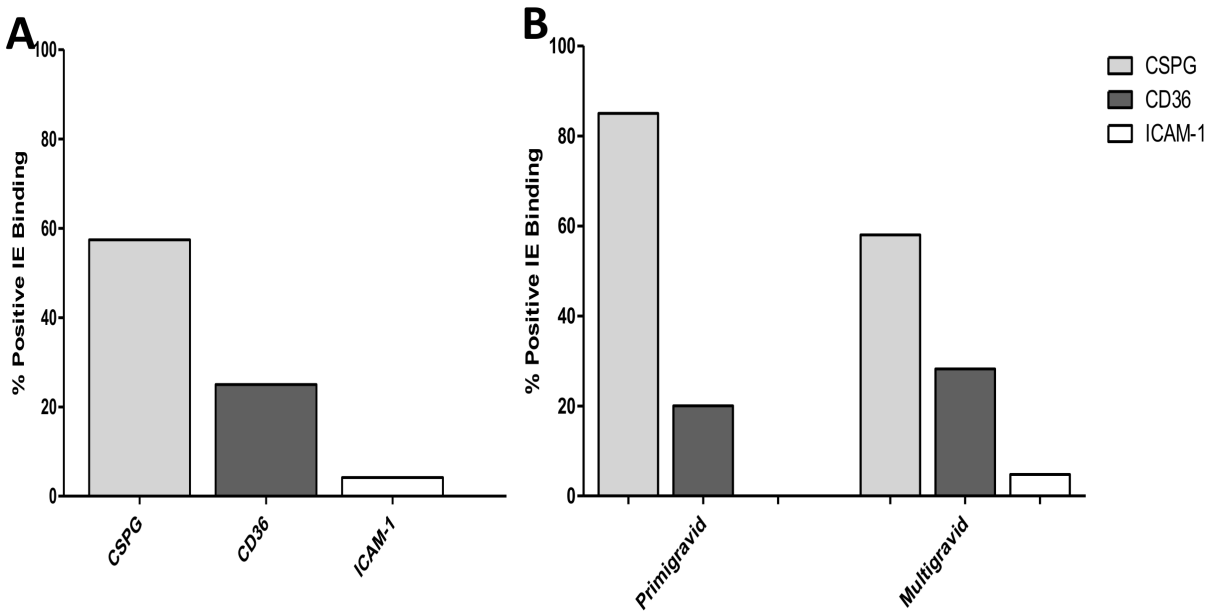

Figure 2. Adhesion profile of isolates from pregnant women. The binding phenotype of isolates was assessed on CSPG, CD36 and ICAM-1 receptors and presented as proportion of "positive" adhesion to each receptor, defined at binding ≥35 IE/mm^2 (A) – The binding profile of all tested isolates and (B) The segregation of the isolates binding profile according to the parity of women.
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isolates from both primi- and multigravidae bound CD36 and ICAM-1 to similar levels (Table 5). Likewise, when the threshold of significant binding was applied, the analysis revealed a trend of parasites from primigravidae to adhere more frequently (OR = 3.2) to CSPG than those from multigravidae, although not strictly significant (*P = 0.11*), possibly due to a lack of power associated to our limited sample size. Although the affinity for CD36 was more advantageous among isolates from multigravidae with an unfavorable linear coefficient of -52.3 among isolates from primigravidae (Figure 2B), this relationship was not observed in our model of logistic regression analysis (OR = 0.64, *P = 0.6*) (Figure 2B). Regarding adhesion to ICAM-1, the logistic regression model failed to converge, due to numerical trouble.

To investigate whether gestational age is associated with a particular binding phenotype of isolates, a cut-off was made at 16 weeks of gestational age to define early (<16 weeks) and late pregnancy (>16 weeks). For ICAM-1 receptor, the mixed models failed to converge. Isolates from late pregnancy bound CSPG to a higher level than those from early pregnancy (*P = 0.03,* Table 4). The opposite was observed with CD36, with a lower binding level of late pregnancy parasites (*P = 0.02*). This lower ability of isolates from late pregnancy to bind CD36 was confirmed also by logistic regression analysis (OR = 0.14; *P = 0.01*). No association was observed between parasite density and binding phenotype of the isolates. In addition, when analysis was done according to the pregnancy outcome, such as the birth weight of the baby and the level of maternal hemoglobin, no relationship was observed with a particular adhesion pattern of IE.

Discussion

The ability of *P. falciparum* IE to bind to CSA is the key factor that mediates placental sequestration and consequently the pathogenesis of malaria during pregnancy. Several studies have described the particular adhesion phenotype that characterizes parasites collected from women during pregnancy [3,23,25,36], demonstrating evidence of a distinct binding ability to CSA shared by such isolates. Most of these studies have focused on isolates collected in late pregnancy [23,25,39], and observations have greatly helped formulate the hypothesis that the parasite ligand mediating adhesion to CSA would represent the main target for potential vaccine development against PAM. However, few studies have investigated the binding phenotypes of parasites infecting pregnant women early in pregnancy, their dynamics throughout pregnancy in relation to VAR2CSA expression, and whether pregnancy-related factors such as parity and gestational age can influence these phenotypes.

Recently, we have demonstrated that infection by CSA-binding isolates occurs in the first trimester of pregnancy [36]. Not all

Table 3. Predominant *var* genes transcripts in PAM isolates adhering to CSA and CD36, *P<0.0001**.

var gene groups	*var ABC*	*var1*	*var2csa*
CSA-adhering isolates[a] (n = 32)	0	12.5	87.5
CD36-adhering isolates[a] (n = 13)	23	46	31

*Fisher Exact test.
[a]adhesion defined as binding ≥35 IE/mm^2.
doi:10.1371/journal.pone.0098577.t003

Table 4. CSA-binding isolates and VAR2CSA surface expression in PAM isolates predominantly transcribing *var2csa* and non-*var2csa* genes.

Dominant *var* gene transcribed	CSA-adhering isolates[a] (n = 32)	VAR2CSA surface detection**	Non or weakly CSA-adhering isolates[b] (n = 22)	VAR2CSA surface detection**
var2csa	28	28	10	10
non-*var2csa*	4	4	12	1

**Significant surface labeling by VAR2CSA antibodies with MFI ratio >1.2.
[a]adhesion defined as binding ≥35 IE/mm^2.
[b]binding level <35 IE/mm^2.
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isolates collected at this stage of pregnancy bound to CSA. In this study, we collected and analyzed the parasites from peripheral blood of pregnant women at different times of pregnancy. In addition to CSA, we have now assessed which of the other receptors (CD36 and ICAM-1) are most commonly preferred by isolates collected throughout pregnancy and investigated whether infection by parasites with an adhesion pattern to these receptors in a particular timing of pregnancy could be associated with poor outcome. Variability in the binding phenotype of isolates was observed throughout pregnancy. Our data highlights the coexistence of parasites with several binding phenotypes in early pregnancy isolates. But, this diversity gradually tightens with gestational age in favor of the CSA binding phenotype. This finding points out the high risk of women infection by parasites with CSA-binding phenotype late in pregnancy, suggesting that isolates with adhesion pattern to other receptor are less involved in the pathogenesis of PAM requiring placental tropism.

However, adhesion to CSA was the most prevalent phenotype displayed by peripheral isolates from pregnant women compared to binding to either CD36 or ICAM-1. Generally, these isolates bound to CSA at higher levels compared to CD36 and ICAM-1. Although the placental tropism of CSA-binding parasites might explain this adhesion preference of the isolates, a possible role of the pre-existing immunity in these women, against CD36- and ICAM-1-binding parasites should be considered. This pre-existing immunity acquired from childhood mainly against CD36- and ICAM-1-binding parasites could be an important filter. As already described [20,25,39], interactions with ICAM-1 were observed with few isolates from pregnant women, while adhesion to CD36 was more frequently observed among isolates from multigravidae (28%) than those from primigravidae (20%). These observations clearly indicate that infections with parasites not adhering to CSA also occur during the pregnancy. Although the importance of these infections in the outcome of pregnancy is unknown, their characterization remains an open issue in the context of pregnancy success in malaria-endemic regions. Such infections are dependent on gestational age, occurring earlier in pregnancy and gradually decreasing with increasing gestational age. It is likely that the generalized immuno-modulation that occurs during pregnancy favors infections with *P. falciparum* regardless of the binding phenotype in primigravid women. The restriction of this phenotype to parasites with a preference for CSA occurs gradually as the placenta grows and becomes increasingly irrigated. It is quite plausible that interventions like IPTp also promote this phenotypic refining by increasing the fitness of placental parasites that will be more preferably selected in subsequent infections. On the other hand, non-CSA binding phenotypes seem to be more common in multigravidae, in whom immunity against CSA-binding parasites is well described [40,41]. The re-emergence of infections with non-CSA binding parasites suggests better control of CSA-binding phenotypes via acquired immunity, thereby restoring the diversity of binding properties observed in non-pregnant individual. However, we did not observe a significant association between a particular adhesion phenotype of isolates and outcomes such as the maternal hemoglobin and birth weight of babies, probably due to the fact that all the infected pregnant women were systematically treated in this study.

Many studies have demonstrated the high susceptibility of women to PAM during the first pregnancy due to the lack of the protective immunity that is acquired following successive pregnancies [1,15,42]. In line with these reports, our data emphasize the high vulnerability of primigravid women to infection by parasites with a CSA-binding phenotype. This increased vulnerability suggests that these parasites that strongly adhere to CSA are those that cause the worst pregnancy outcomes, as supported by their high frequency in primigravidae, who are the most at risk of malaria consequences during pregnancy [1,3,42].

On the other hand, measurement of the transcription level of *var2csa* compared to other *var* genes were performed, and its expression as a protein on the surface of IE was assessed by use of specific anti-VAR2CSA antibodies. High transcription level of *var2csa* was observed among PAM-isolates, in agreement with prior reports that have identified this gene as being specifically highly transcribed in isolates from pregnant women. Although *var2csa* transcripts were detected in most isolates, infections with parasites that dominantly transcribed other *var* genes were observed. Most of these isolates preferentially bound to CD36 and/or ICAM-1. The binding preference to CSA was exclusively observed among isolates in which the transcription of *var2csa* was clearly dominant over that of the other *var* genes [43,44]. Co-expression of multiple *var* genes, due to clonal phenotypic variation of parasites and to the multiplicity of infections, might explain the fact that some isolates were able to bind to more than one receptor.

The positive association between the surface expression of VAR2CSA and ability of IE to bind CSA supports previous reports indicating VAR2CSA is the main protein involved in this interaction. However, some few isolates did not bind CSA whilst simultaneously exhibiting both a marked predominant transcription of *var2csa* and a specific labeling of VAR2CSA on the IE surface. A plausible explanation might be that the immobilized receptor binding-assay does not fully reproduce the physiological conditions mediating *in vivo* interactions, due to differences in protein conformation and localization. However, previous studies have demonstrated a variable ability of placental isolates to bind CSA [3,20]. Further investigations using cell-based methods under flow conditions are needed to better characterize these low CSA-binding isolates, and to assess whether other factors or proteins are involved in the CSA-binding process. Conversely, in some isolates that showed binding to CSA, predominant transcription of other *var* genes instead of *var2csa* was noted. The polyclonal nature of

Table 5. Parity and gestational age dependence of PAM-isolates binding properties.

	Linear regression		Logistic regression	
	Coefficient* (95% CI)	**p**	**OR (95% CI)**	**p**
Primigravidae and multigravidae[a]				
CSPG	118.1 (−3.01–239.38)	0.05	3.2 (0.76–13.24)	0.11
CD36	−52.36 (−193.09–88.37)	0.45	0.64 (0.12–3.48)	0.60
ICAM-1	0.93 (−5.46–7.33)	0.76	CD	
Early and late pregnancy[b]				
CSPG	128.74 (16.73–240.75)	0.03	2.13 (0.62–7.37)	0.23
CD36	−117.24 (−216.62–17.87)	0.02	0.14 (0.03–0.61)	0.01

*Difference in the mean of adhesion level between the considered and reference classes.
[a]Reference class: Multigravidae.
[b]Reference class: Early pregnancy (<16 weeks of gestation).
OR = Odd ratio; CD = convergence default.
doi:10.1371/journal.pone.0098577.t005

pregnant women infections may partially explain this observation. It is also possible that the dominant transcribed *var* genes in these isolates might encode for particular adhesion phenotype which was not explored in this study. However, a possible role of other non-VAR2CSA parasite proteins expressed on the surface of IE in the interaction with the CSA is not to be excluded. Further studies are still needed to make this clarification.

In summary, the data presented here are of capital importance in the context of VAR2CSA-based vaccine development. Actually the expression of VAR2CSA appears as the major feature shared by the *P. falciparum* parasites infecting pregnant women. These data suggest a major interest in VAR2CSA variants that express a strong adhesion ability to CSA as a critical aspect to be considered in the ongoing effort of vaccine development.

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Author Contributions

Conceived and designed the experiments: JD NTN. Performed the experiments: JD SS A. Moussiliou. Analyzed the data: JD GC NTN. Contributed reagents/materials/analysis tools: A. Massougbodji CHH PD. Wrote the paper: JD PD NTN.

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Article III: The NTS-DBL2X region of VAR2CSA induces cross-reactive antibodies that inhibit adhesion of several Plasmodium falciparum isolates to chondroitin sulfate A

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Plusieurs travaux, relayés par les deux articles précédents, ont permit de conforter le rôle clé de VAR2CSA dans la pathologie du paludisme placentaire ainsi que son intérêt comme une cible privilégiée dans le cadre d'un développement vaccinal contre le paludisme associé à la grossesse. Toutefois, plusieurs défis se sont avérés dans la faisabilité d'un tel vaccin. L'un d'eux reposait sur la taille de cette grande protéine multi-domaine qui complique sa production dans les conditions GMP d'antigènes à usage vaccinale. Cette difficulté, conjuguée à la probable nécessité de combiner un certain nombre réduit de variants dans une formulation capable d'induire des anticorps avec un large spectre d'activité, a motivé la recherche de fragments plus courts de la protéine possédant des épitopes fonctionnelles.

Dans ce travail, 13 constructions tronquées de VAR2CSA représentant un ou plusieurs domaines qui se chevauchent ont été réalisées à partir de la séquence *var2csa* de la lignée parasitaire FCR3. Une expérience pilote de vaccination génétique réalisée chez la souris et le lapin, avec ces constructions insérées dans des plasmides pVAX1, a montré que tous les immunogènes étaient capables d'induire des anticorps polyclonaux après électro-transfert de plasmides recombinants par voie intramusculaire (avec des titres ELISA significativement élevés ($>1E^{+05}$). Pour les plasmides contenant des inserts de plus de 3000 pb de la séquence codante, l'efficacité de la réponse immunitaire humorale a nécessité l'utilisation d'une séquence dont les codons ont été optimisés afin de faciliter l'expression (GenBank accession no. GU249598). Le plasmide optimisé, codant pour la totalité de la partie extracellulaire, a ainsi induit une forte réponse anticorps chez tous les animaux vaccinés (souris et lapins). Les anticorps produits contre toutes les constructions de VAR2CSA ont pu réagir avec la protéine native exprimée à la surface des EI. Cette réactivité mesurée par cytométrie en flux a été obtenue sur des EI de la lignée parasitaire homologue FCR3, sélectionnés pour adhérer à la

CSA (FCR3-BeWo). Aucune réactivité n'a été observée pour la souche non-sélectionnée sur CSA.

L'analyse du pouvoir inhibiteur des anticorps polyclonaux ainsi produits sur l'adhérence des érythrocytes infectés au récepteur CSA a permis non seulement de confirmer le fort pouvoir inhibiteur des anticorps développés contre la partie extracellulaire de VAR2CSA, mais aussi d'identifier un fragment minimal de celle-ci dans l'extrémité N-terminal. La construction NTS-DBL2X concentre les épitopes anti-adhérence. Ce fragment de VAR2CSA a permis d'obtenir des anticorps hautement inhibiteurs montrant une activité similaire à celle des anticorps induits par le fragment représentant la totalité de la partie extracellulaire. En outre, la même activité inhibitrice avait été observée sur une lignée parasitaire hétérologue CSA-adhérents, la lignée HB3. Les IgG spécifiques purifiées (par affinité sur la protéine NTS-DBL1X-ID1-DBL2X) à partir des sérums de femmes multigestes exposées du Bénin étaient capables de reconnaitre la surface des érythrocytes infectés par FCR3 CSA-adhérents. Ces anticorps pouvaient aussi inhiber complètement l'adhérence de ces EI à la CSA à une concentration de 0,5mg.mL^{-1}. Ceci conforte davantage l'intérêt de cette région de la protéine dans l'induction des anticorps anti-adhérence.

En utilisant la cytométrie il a été clairement démontré que les anticorps murins anti-NTS-DBL2X reconnaissent spécifiquement la surface des érythrocytes parasités provenant de femmes enceintes. Des 30 isolats testés (6 isolats placentaires et 24 obtenus du sang périphérique), 21 ont été marqués par ces anticorps alors qu'aucun des 5 isolats d'enfants testés n'a été marqué. L'évaluation des propriétés anti-adhérences sur ces isolats de patients a été réalisée sur 15 isolats (14 provenant du sang périphérique et 1 du sang placentaire). Un effet inhibiteur des IgG anti-NTS-DBL2X a été observé sur 12 des 15 isolats.

Ce travail met clairement en évidence l'intérêt de la région NTS-DBL2X de VAR2CSA dans le mécanisme d'adhérence des EI à la CSA et son rôle dans l'acquisition d'anticorps anti-adhérence chez les femmes multigestes.

The NTS-DBL2X Region of VAR2CSA Induces Cross-Reactive Antibodies That Inhibit Adhesion of Several *Plasmodium falciparum* Isolates to Chondroitin Sulfate A

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Background. Binding to chondroitin sulfate A by VAR2CSA, a parasite protein expressed on infected erythrocytes, allows placental sequestration of *Plasmodium falciparum*–infected erythrocytes. This leads to severe consequences such as maternal anemia, stillbirths, and intrauterine growth retardation. The latter has been clearly associated to increased morbidity and mortality of the infants. Acquired anti-VAR2CSA antibodies have been associated with improved pregnancy outcomes, suggesting a vaccine could prevent the syndrome. However, identifying functionally important regions in the large VAR2CSA protein is difficult.

Methods. Using genetic immunization, we raised polyclonal antisera against overlapping segments of VAR2CSA in mice and rabbits. The adhesion-inhibition capacities of induced antisera and of specific antibodies purified from plasma of malaria-exposed pregnant women were assessed on laboratory-adapted parasite lines and field isolates expressing VAR2CSA. Competition enzyme-linked immunosorbent assay (ELISA) was employed to analyze functional resemblance between antibodies induced in animals and those naturally acquired by immune multigravidae.

Results. Antibodies targeting the N-terminal sequence (NTS) up to DBL2X (NTS-DBL2X) efficiently blocked parasite adhesion to chondroitin sulfate A in a manner similar to that of antibodies raised against the entire VAR2CSA extracellular domain. Interestingly, naturally acquired antibodies and those induced by vaccination against NTS-DBL2X target overlapping strain-transcendent anti-adhesion epitopes.

Conclusions. This study highlights an important step achieved toward development of a protective vaccine against placental malaria.

Placental malaria (PM) is an important cause of maternal anemia, stillbirth, and low birth weight children in Africa. In this syndrome, *Plasmodium falciparum* infected erythrocytes (IEs) accumulate in the placenta by binding to the chondroitin sulfate A (CSA) moiety of placental intervillous chondroitin sulfate proteoglycan (CSPG). This binding is mediated by a member of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family named VAR2CSA, which is expressed on the surface of IEs [1–3]. Reduced susceptibility to placental malaria is observed in women who have acquired immunoglobulin G (IgG) that blocks adhesion of IE to the placental receptors through several malaria-exposed pregnancies [4–6]. Increased levels of those antibodies have been associated with improved pregnancy outcomes, suggesting their importance in immunity to PM [5, 7, 8]. The implication of VAR2CSA in the expression of the CSA-adhesion phenotype by IE has

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clearly been demonstrated [9], and accumulated evidence shows that naturally acquired antibodies against VAR2CSA play a major role in the protective immunity to PM [10, 11]. The *var2csa* gene is present in almost all *P. falciparum* isolate genomes as well as in the close relative *Plasmodium reichinovii*, and it encodes a high molecular weight, polymorphic protein of about 350 kDa consisting of 6 Duffy binding–like (DBL) domains, a cysteine-rich interdomain region (CIDR)–like domain, and 2 interdomain regions [12, 13]. The large size and the interclonal variation among *var2csa* alleles have been of major concern in the feasibility of an effective VAR2CSA-based vaccine. Experimental vaccination of animals with single-domain VAR2CSA recombinant proteins induced antibodies that at various degrees could inhibit CSA adhesion of erythrocytes infected by different laboratory-adapted clones of *P. falciparum* [14, 15].

The full-length extracellular part of the molecule has recently been expressed for the first time in eukaryotic systems [16, 17], and antibodies induced against this construct showed a very high anti-adhesion IgG titer. However technological constraints in the optimal production of such a large antigen question the use of full-length VAR2CSA in vaccine development. Defining smaller parts of the molecule with functionally important antibody epitopes shared among most VAR2CSA variants, ie, inducing antibodies that inhibit CSA binding of IEs is still of much interest in a rational way of developing a VAR2CSA-based vaccine.

In this study, we investigated the possibility of identifying such functionally important VAR2CSA regions that can induce IgG with high adhesion inhibitory capacity. Using intramuscular plasmid DNA electrotransfer, we showed that antibodies induced against a specific N-terminal region of VAR2CSA, the NTS-DBL2X, can efficiently block parasite binding to CSA at a similar level to that of IgG induced against the full-length extracellular part of the molecule. This work highlights an important achievement toward development of a protective vaccine against placental malaria.

METHODS

Ethics Statement

The study was approved by the Comité Consultatif de Déontologie et d'Ethique of the Research Institute for Development (France); the ethical committee of the Ministry of Health, Senegal; and the ethics committee of Health Science Faculty, University of Abomey-Calavi (Benin). All procedures complied with European and national regulations.

All procedures regarding animal immunizations complied with European and national regulations. Ethics statement details are given as Supplementary material.

Parasites and Human Plasma

In this study, we used in vitro–propagated *P. falciparum* parasites FCR3 and HB3 grown in O+ erythrocytes without human serum as described [18]. We tested antibody reactivity with IEs on unselected cultures and cultures selected for IE adhesion to CSA. We selected cultures following several panning on the choriocarcinoma cell line BeWo as described [19].

Primary field *P. falciparum* isolates and plasma samples were collected from a cohort of pregnant women enrolled in the STOPPAM project in the district of Comé at the Mono province, located 70 km West from the economical capital of Benin, Cotonou [20]. The isolates were obtained either from the peripheral blood of children below the age of 5 years ($n = 5$) and pregnant women ($n = 24$), or from placental blood at delivery ($n = 6$). Peripheral blood isolates were maintained in vitro for ≤48 hours before testing.

Plasma samples from a previous study conducted in Senegal were also used [8].

Animal Immunization and Antibody Screening

DNA sequences encoding single and multiple domains of VAR2CSA protein were cloned into a pVax1 vector derivative and fused to the mEPO signal sequence as already described [21]. We anesthetized 6-week-old Swiss female mice by intraperitoneal injection of 0.3 mL of a mix of ketamine (8.66 mg/mL) and xylazine (0.27 mg/mL) in 150 mM sodium chloride. 40 μg of plasmid DNA was injected into the tibial cranial muscle. Transcutaneous electric pulses (200 V/cm, 20 ms, 5Hz) were then applied at each side of the leg [21]. New Zealand rabbits were anesthezied by intramuscular injection of a ketamine–xylazine mix, 300 μg of plasmid was injected in 5 sites of each *longissimus dorsi* muscle, and electrical pulses (120V/cm, 20 ms, 5Hz) were applied. We performed mice protein immunizations by intraperitoneal injection of a recombinant protein–Alugel mix. Animals were immunized at days 0, 30, and 60, and antisera were collected at days 45 and 75. IgG serum titres were determined by enzyme-linked immunosorbent assay (ELISA) tests using each recombinant DBL domain. Animal immunization and antibody screening details are given as SI text.

IgG Preparation

We manually purified total IgG from final bleed mouse or rabbit sera on a Hi-Trap Protein G High Pressure (HP) column according to manufacturer's recommendations (GE Healthcare). We affinity-purified construct-specific IgG from plasma pools of women exposed to placental malaria and exposed males using HiTrap N-Hydroxy-Succinimide (NHS)-activated HP columns (GE Healthcare, http://www.gehealthcare.com) on which the corresponding recombinant protein was coupled following the manufacturer's instructions.

Antibody Reactivity With *Plasmodium falciparum* Laboratory Lines and Field Isolates

In vitro propogated *P. falciparum* parasites FCR3 and HB3 were repeatedly panned on the human choriocarcinoma cell line BeWo as described [19]. We analyzed the derived CSA-adhering

IEs (FCR3--BeWo, HB3--BeWo) and 35 primary field *P. falciparum* isolates collected at Comé, southwestern Benin [22] to determine reactivity of all generated antibodies. We used flow cytometry (FACS Calibur) to test the serum reactivity of vaccinated animals to the surface of the IE as previously described [23].

Protein Expression, Purification, and Evaluation

The NTS-DBL2X region of the *var2csa* gene from FCR3 parasite line (synthetic gene) was cloned from amino acid N9 to A864 into the baculovirus vector pAcGP67-A (BD Biosciences) upstream of a histidine tag in the C-terminal end of the construct. This construct was expressed and purified. Protein expression, purification, and evaluation details are given as SI text.

Specific recognition of the purified protein was evaluated in ELISA using plasma samples from pregnant women of Benin and Senegal, unexposed pregnant French women, and malaria-exposed children (Senegal) and men (Benin and Senegal).

Inhibition of IE Binding to CSPG by Specific IgG

The static assays employed to evaluate the capacity of the antibodies to interfere with CSA-specific adhesion of IEs was described in detail elsewhere [24]. In this assay, plates were coated overnight at 4°C with 20 μL of ligand: 1% bovine serum albumin (BSA), 5 $\mu g.mL^{-1}$ decorin: CSPG (Sigma) or 50 $\mu g.mL^{-1}$ bovine CSA (Sigma) diluted in phosphate-buffered saline (PBS). Each spot was subsequently blocked with 3% BSA in PBS for 30 minutes at room temperature (RT). Late stage–infected IEs were also blocked in BSA/RPMI for 30 minutes at RT. Parasite suspensions adjusted to 20% parasite density were incubated with serum (1:5 final dilution), purified IgG (0.01 $mg.mL^{-1}$ to 1 mg/mL final concentration), or 500 $\mu g.mL^{-1}$ soluble CSA for 30 minutes at RT before they were allowed 15 minutes at RT to bind to ligand. Nonadhering cells were removed by an automated washing system. Spots were fixed with 1.5% glutaraldehyde in PBS and adhering IEs were quantified by microscopy.

Competition ELISA

Prior to competition of ELISA, the anti–NTS-DBL2X IgG titre was determined in plasma pools composed of samples from exposed multigravid women from Benin, DNA-vaccinated rabbits, and protein-immunized mice (plasma pools from D75). Microtiter plates were coated with recombinant NTS-DBL2X (0.5 $\mu g.mL^{-1}$ in PBS). Competition ELISA details are given as SI text.

RESULTS

Plasmid DNA Immunization Induced High Titre Surface Reactive Antibodies

A total of 13 plasmids representing single and overlapping multiple domains of VAR2CSA from the FCR3 (Figure 1*A*; Table 1) parasite line were constructed and used for immunization. All immunizations with single- to triple-domain constructs of VAR2CSA induced polyclonal antibodies with a high ELISA titre ($>1\ E^{+05}$) following intramuscular plasmid electrotransfer. However, for plasmids containing more than 3000 base pairs of coding sequence, effective humoral immune response in all vaccinated animals, both mice and rabbits required the use of a codon-optimized sequence (GenBank accession no. GU249598). Although all single domains and multidomains of VAR2CSA could induce antibodies reacting with native VAR2CSA on the surface of the CSA-adhering erythrocytes infected with the homologous FCR3, constructs containing DBL1x, DBL2x, DBL5e, and DBL6e were the most efficient in inducing surface reactive antibodies (Figure 1*B*). None of the polyclonal anti-VAR2CSA antisera recognized the erythrocytes infected with the non-CSA adherent FCR3 parasite line.

Antibodies Induced Against VAR2CSA Abrogate Binding of Infected Erythrocytes to Chondroitin Sulphate Proteoglycan

The petri dish–based static binding assay was used to screen sera for the ability to inhibit parasite binding to CSA. Of all the FCR3 VAR2CSA regions tested, only sequences located between the NTS and the DBL3X appeared to induce inhibitory antibodies (Figure 1*B*). Highly inhibitory antibodies were obtained with the full-length extracellular VAR2CSA construct, which totally abrogated binding. Interestingly, similar inhibition was seen with sera from animals (both mice and rabbits) vaccinated with the NTS-DBL2X construct. In addition, we investigated the inhibitory activity of sera from NTS-DBL2X vaccinated animals on a heterologous parasite line; the CSA adherent HB3 line. The same pattern of inhibition was observed (Figure 2*A*)

To confirm that the inhibition observed with NTS-DBL2X antiserum was mediated by IgG, we purified IgG and tested for binding inhibition activity. The purified IgG recognized the surface of BeWo-selected FCR3 IEs (Figure 2*B*). The IgG inhibited 100% of the binding of infected erythrocytes to CSA at a concentration of 0.5 $mg.mL^{-1}$ (Figure 2*D*).

Antibodies Induced Against NTS-DBL2X Specifically Recognized Isolates From Pregnant Women

Flow cytometry analysis clearly demonstrated that murine anti–NTS-DBL2X antibodies specifically recognize the surface of PM parasites among the field isolates. In this study, we used flow cytometry to analyze 35 isolates, which comprised 24 peripheral blood isolates from pregnant women, 6 placental isolates, and 5 peripheral isolates from children. Twenty-one of the 24 peripheral blood isolates from pregnant women were recognized by polyclonal murine antibodies, whereas none of the 5 children isolates tested were labeled.

Of the 21 isolates from pregnant women that reacted with anti–NTS-DBL2X antibodies by flow cytometry, 16 showed specific adhesion to CSPG, whereas 5 isolates did not bind. Among the 3 peripheral blood isolates that were not labeled in flow cytometry, 2 bound to CSPG but their interaction could not be abrogated by soluble CSA, and 1 isolate did not bind.

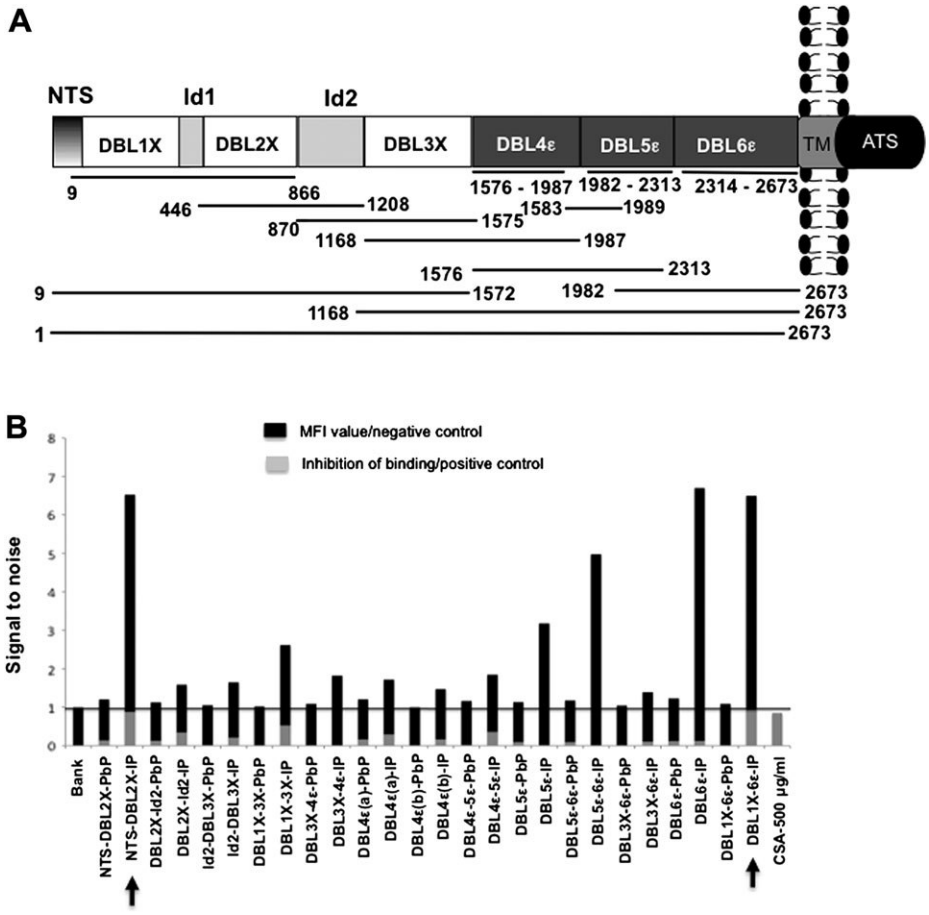

Figure 1. Surface reactivity and anti-adhesion capacity of mice antisera to various VAR2CSA constructs. *A,* Schematic representation of the 13 overlapping VAR2CSA constructs made in a pVAX1 derivative vector. *B,* Blood samples before immunization and from full bleeds at D75 were pooled for each group of 5 animals to constitute the prebleed pool (PbP) and immune pool (IP) respectively. The flow reactivity shown (black filled histograms) for each pool is defined as the median fluorescence intensity (MFI) ratio (MFI test/MFI negative control). The negative controls were stained only by secondary fluorescein isothiocyanate (FITC)–conjugated antimouse antibody. Binding inhibition of infected erythrocytes to decorin (bovine chondroitin sulfate proteoglycan [CSPG]) was measured using serum in a 1:5 dilution, grey-filled histograms. The degree of inhibition was defined as (1 – [the ratio of bound infected erythrocytes with test serum/bound infected erythrocytes without serum]).

We further processed in binding inhibition assay 15 samples containing sufficient amounts of parasites. These comprised 14 isolates from peripheral blood samples and 1 placental isolate. The binding to CSA of 12 of the 15 pregnant women isolates tested was highly inhibited by specific anti–NTS-DBL2X sera (Table 2).

Animals Immunized With Recombinant NTS-DBL2X or DNA Electrotransfer Produced Antibodies of Similar Specificity

Murine polyclonal antibodies induced either by recombinant protein or plasmid DNA of NTS-DBL2X showed similar reactivity. The reactivity to the erythrocyte surface and inhibitory activity on binding to CSA were similar on BeWo-selected FCR3 infected erythrocytes. The inhibitory activity was compared in dilution series of sera from mice immunized with either the full-length construct or NTS-DBL2X (both DNA and protein immunization). Down to the dilution 1:100, sera from mice vaccinated with full-length DNA construct or recombinant NTS-DBL2X totally inhibited binding of infected erythrocytes (Figure 3*A*). The inhibition capacity of the serum samples following plasmid DNA immunization with the full-length construct or by protein vaccination with NTS-DBL2X was seen at subsequent dilutions, these sera were diluted 1:5000 before inhibition vanished (Figure 3*A*). This observation clearly strengthens the importance of the NTS-DBL2X part of VAR2CSA in eliciting adhesion-inhibitory antibodies by vaccination.

Antibodies Induced in Animals by Vaccination With NTS-DBL2X Target the Same Epitopes as Naturally Acquired Antibodies

The recombinant NTS-DBL2X produced in insect cells was recognized by plasma from malaria-exposed pregnant women from Benin and Senegal in a parity-dependent manner (Supplementary Figure 1). This NTS-DBL2X was used in competition ELISA to analyze the target epitopes among

Table 1. VAR2CSA Constructs of *Plasmodium flaciparum* Parasite FCR3

VAR2CSA construct	Amino acid position
NTS-DBL2X	9–866
DBL2X-Id2	446–1208
Id2-DBL3X	870–1575
DBL3X-4ε	1168–1987
DBL4ε(a)	1576–1987
DBL4ε(b)	1583–1989
DBL4ε-5ε	1576–2313
DBL5ε	1982–2313
DBL5ε-6ε	1982–2673
DBL6ε	2314–2673
NTS-DBL3X	9–1572
DBL3X-DBL6ε	1168–2673
NTS-DBL6ε	1–2673

The antigens cloned from FCR3 VAR2CSA used for immunizations are described. Antigen boundaries are indicated as start and stop amino acids from the coding sequence.

antibodies induced in animals by plasmid DNA immunization and protein immunization, as well as the naturally acquired antibodies against the NTS-DBL2X part of VAR2CSA in pregnant women. A mutual inhibition pattern was observed in the ability of all 3 antisera to recognize the recombinant NTS-DBL2X protein. The inhibition pattern between sera from DNA immunizations and protein immunizations was concentration- dependent (Figure 3*B*). A similar inhibition was observed when antibodies in a human plasma pool from exposed multigravidae competed with specific antisera from rabbits (Figure 3*C*).

The Naturally Acquired Human IgG Against VAR2CSA NTS-DBL2X Inhibit Adhesion of Infected Erythrocytes to CSA

Plasma samples from women included in the STOPPAM project are routinely analyzed for anti-adhesion capacity on the FCR3-BeWo and HB3-BeWo parasite lines. The recombinant NTS-DBL2X protein was used to affinity-purify IgG from plasma of malaria-exposed Beninese pregnant women (selected for having a high anti-adhesion activity on CSA-binding parasite lines). Interestingly, naturally acquired antibodies targeting the NTS-DBL2X of VAR2CSA demonstrated anti-adhesion activity. This activity was shown both on FCR3-BeWo and HB3-BeWo parasite lines, with a clear concentration-dependent effect of purified IgG (Figure 4). This is the first time that naturally acquired antibodies to a specific VAR2CSA region have been shown to inhibit *P. falciparum*–infected erythrocytes binding to CSA.

DISCUSSION

Molecular details of the interaction of the *P. falciparum* ligand VAR2CSA with the placental receptor CSA are currently not well delineated, but recent studies suggest that the binding site depends on a higher-order architecture in which DBL domains and the interdomain regions of VAR2CSA fold together to form a ligand-binding pocket [16, 25]. However, polyclonal antibodies induced by immunization with the recombinant extracellular part of VAR2CSA highly inhibit binding of IEs to CSA in vitro [16]. This suggest that protective immunity to placental malaria acquired over a few pregnancies in areas of intense *P. falciparum* transmission that correlates with levels of anti-adhesion antibodies [7] is mostly mediated by anti-VAR2CSA IgG. Nevertheless, a recent work reports that immunization with full-length VAR2CSA did not induce potent cross-inhibitory antibodies [26]. This is surprising, but, as indicated by the authors, it could relate to the recombinant protein preparation (FCR3 allele in baculovirus insect cells versus 3d7 allele in mammalian cells), the animal species immunized, the adjuvant employed (Freund complete/incomplete adjuvant vs titer-max), or simply the size and complexity of full-length VAR2CSA immunogens.

Although antibody response may directly inhibit IE adhesion placenta, it also might be implicated in opsonization [27, 28].

VAR2CSA thus appears as an important candidate for vaccine development, however sequence analyses among parasites have shown that it is a polymorphic protein composed of alternating areas of substantial interclonal polymorphism [12, 13]. The rationale for developing an effective VAR2CSA-based vaccine against placental malaria thus requires definition of VAR2CSA areas containing functionally important epitopes that transcend this interclonal diversity. In this study, full-length and truncated VAR2CSA constructs were studied for their ability to induce adhesion-inhibitory antibodies.

The DNA vaccine technology that has proven efficient on various pathogens and tumor antigens [29] was successfully achieved here with the *P. falciparum var2csa* gene. The resurgence in interest for such concept observed in the last few years is due to several technical improvements, such as codon optimization strategies, novel formulations, and more-effective delivery approaches. The delivery of electrical pulses after intramuscular plasmid DNA injection particularly enhanced DNA uptake and resulted in a stronger and more specific humoral response when the antigen was fused to a leader sequence [21]. Several clinical trials based on this approach are currently being conducted in the fields of cancer and infectious diseases (http://clinicaltrials.gov/ct2/results%3Fterm=electroporation); 1 of the trials that started in July 2010 targets *Plasmodium falciparum* malaria.

In this study, a strong immune response was obtained both in mice and rabbits vaccinated with VAR2CSA genetic fragments that were fused to the mEPO leader sequence. Interestingly, all antibodies induced were able to recognize the native protein expressed on the surface of erythrocytes infected with the homologous FCR3 parasite line. In line with data previously reported by Khunrae et al [16], the plasmid encoding the full-length extracellular part of the protein induced a robust humoral response

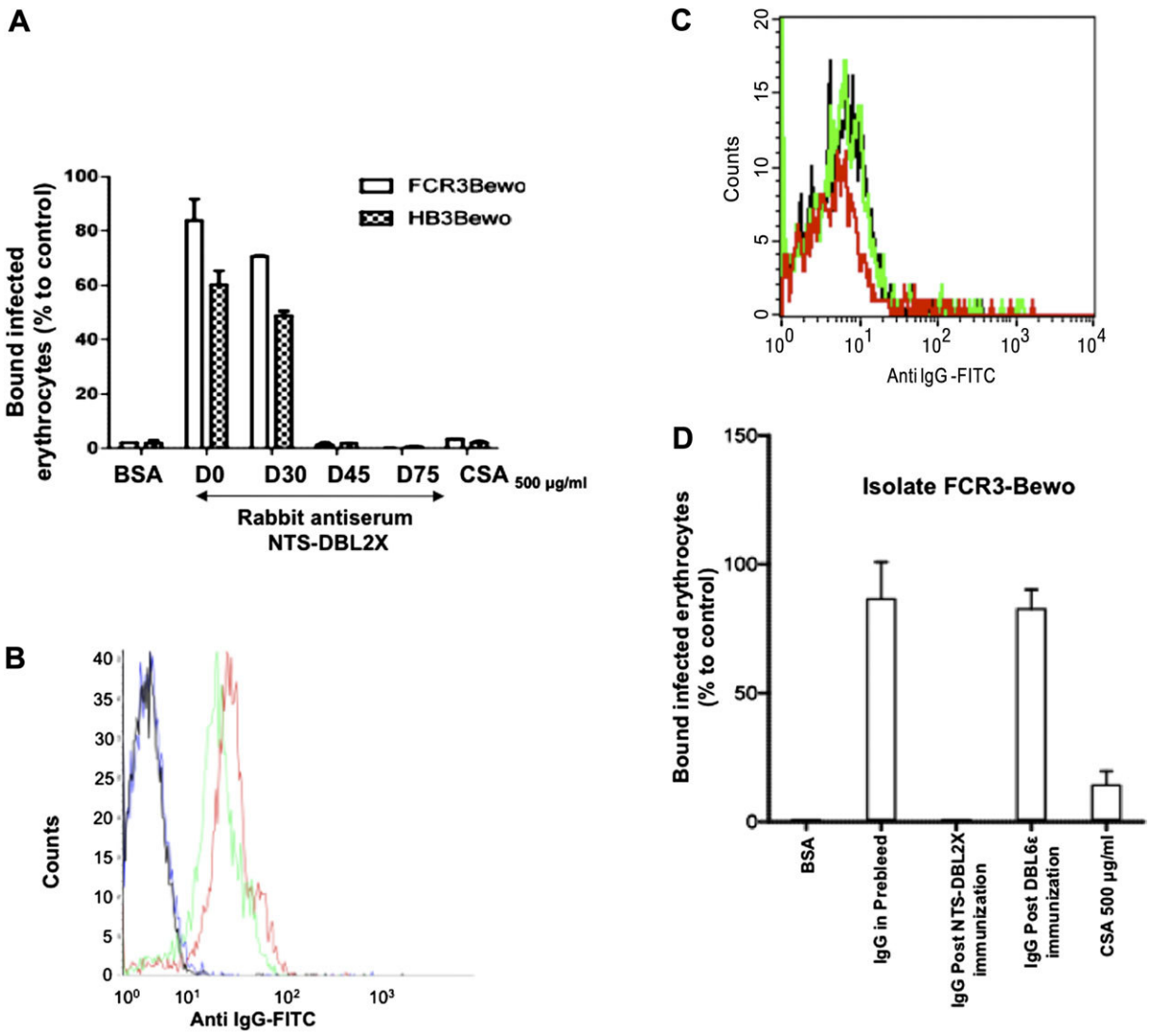

Figure 2. NTS-DBL2X induces adhesion-inhibitory immunoglobulin G (IgG) in distinct animal species. *A,* 2 rabbits immunized with plasmids encoding the NTS-DBL2X of the FCR3 *var2csa* variant acquired anti-adhesion antibodies to both FCR3–BeWo and HB3-BeWo infected erythrocytes (IEs) as from the second immunization. Purified IgG induced against the NTS-DBL2X region (green line) like that induced against DBL6e (red line) recognize native VAR2CSA on the surface of FCR3-BeWo IEs (*B*) but not unselected FCR3 IEs (*C*). IgG purified from animals before vaccination did not label the surface of FCR3-BeWo IEs (blue lines) like the negative control (black line). *D,* The purified anti–NTS-DBL2X IgG specifically inhibit binding of FCR3-BeWo IEs to chondroitin sulfate proteoglycan (CSPG).

that completely blocked IE binding to CSPG. However, the major finding of this study is that a shorter construct of the N-terminal moiety of VAR2CSA corresponding to NTS-DBL2X was able to induce high-potency antibodies with similar inhibitory capacity as those elicited against the full-length VAR2CSA. Moreover, competition ELISA analysis revealed that antibodies raised by experimental immunization (plasmid DNA or purified recombinant protein) or those naturally acquired by pregnant women to this particular region of VAR2CSA predominantly target similar epitopes. This result is in line with others that reported that pregnant women do acquire cross-reactive antibodies [30, 31]. This suggests that vaccination may reproduce, at least partially, natural acquired immunity against placental malaria.

Recombinant NTS-DBL2X expressed in insect cells was specifically recognized by sera from malaria-exposed women in a parity-dependent manner, supporting the fact that this recombinant protein exhibits important targets of the immune response against VAR2CSA. Murine polyclonal antibodies raised against this construct from the FCR3 parasite strain stained the surface of most isolates from pregnant women of Benin. Remarkably, antibodies raised against a single variant of NTS-DBL2X showed consistent inhibitory activity against several isolates originating from pregnant women. The binding of IEs to CSPG/CSA in isolates from 12 of the 15 pregnant women tested was inhibited by more than 50%. This highlights the existence of functionally important

Table 2. Adhesion Inhibitory Capacity of Specific Antibodies Induced Against NTS-DBL2X on *Plasmodium falciparum*-Infected Erythrocytes From Naturally Infected Pregnant Women in Benin

Isolates	Bound IE/mm2 on BSA	Bound IE/mm2 on CSPG	MFI (Ratio to negative control)	% Prebleed inhibition	% anti–NTS-DBL2X inhibition	% CSA inhibition
CM0425	1	1035	3.2	0.00	41.75	92.21
WP0182	2	339	3.8	23.92	74.75	94.16
CM0375	5	63	1.6	0.00	51.32	94.23
WP0140	2.5	244	1.8	16.36	61.65	98.98
WP0161	1	1301	21.0	32.26	87.34	90.75
WP0168	2	473	6.4	0.00	42.35	94.16
CM0437	1	736	4.1	29.89	94.56	93.34
WP0200	4.5	185	1.4	0.00	31.79	68.29
CM445	1.5	218	2.2	28.69	76.27	87.62
AK366	0.5	357	4.3	15.91	63.35	95.99
AK357	2.5	337	1.5	20.66	58.04	91.12
WP203	1	161	4.2	31.26	82.68	85.87
1MH016	2	178	2.0	16.48	53.92	85.04
1MMCH	2.5	394	3.1	1.89	72.87	86.48
CM307	1	623	5.9	17.83	89.45	96.30

Antibody interference with the binding of 15 *P. falciparum-infected* erythrocytes collected from pregnant women to CSPG is shown. Control binding to BSA and competition of binding to CSPG with soluble CSA are shown for each parasite. The surface reactivity of induced antibodies is shown on each IE. The binding inhibitory activity of prebleed (pool of mice sera collected before immunization at day 0) is shown beside that of the corresponding immune serum (pool of sera collected at day 75 after the first immunization). The identification numbers of the parasites tested are shown. Abbreviations: BSA, bovine serum albumin; CSA, chondroitin sulfate A; CSPG, chondroitin sulfate proteoglycan; IE, infected erythrocyte; MFI, median fluorescence intensity.

epitopes within this region of VAR2CSA that are shared by most placenta-sequestering *P. falciparum* isolates. However, all isolates were not inhibited as a probable consequence of antigenic polymorphism. Possible mechanisms of action include that anti–NTS-DBL2X antibodies inhibit IE adhesion to CSPG/CSA by blocking a single, unique CSA-binding site exhibited in the quaternary structure of VAR2CSA, or by modifying the assembly of such high-ordered structure mediating the binding of native VAR2CSA to CSA [15].

A recent work has indicated that antibodies directed against a DBL1X-DBL3X VAR2CSA construct induced inhibitory antibodies [32]. In our system, a lower inhibitory activity was seen with antibodies against the larger fragment NTS-DBL3X, suggesting that this construct, with our defined boundaries, does not accurately reproduce the critical antibody epitopes present in the native molecule or that the focus of the response is predominantly directed toward epitopes that are distant from the functional site. The results presented here clearly indicate that antibody recognition of just a few VAR2CSA variants containing key epitopes might be sufficient to markedly affect the binding of VAR2CSA-expressing IEs to CSA.

Of particular interest, maternal antibodies purified with the recombinant NTS-DBL2X reacted with both -BeWo-selected FCR3 and HB3 strains and showed high inhibitory activity on these 2 distinct parasite lines. This indicates that the inhibitory properties of anti-VAR2CSA antibodies observed in the current study are of biological significance in the acquired immune protection to placental malaria. It was recently shown that some VAR2CSA-specific human monoclonal IgG from *P. falciparum*–exposed women can exhibit some moderate degree of adhesion inhibition that increases with their combination [23]. To the best of our knowledge, this study clearly shows functional evidence on a specific area of VAR2CSA that is a target of significant naturally acquired anti-adhesion antibodies.

Antibodies induced in rats against the VAR2CSA DBL4ε have previously been shown to possess adhesion-inhibitory activity [15]. Those antibodies were able to inhibit CSA-binding of *P. falciparum* IE from pregnant women [33]. No inhibitory activity with antibodies against DBL4ε was found in the current study. It is possible that such antibodies cannot be obtained in mice and that acquisition of adhesion-inhibitory antibodies to this area of *var2csa* is host dependent. Our data nevertheless still support the hypothesis that simultaneous targeting of epitopes in different VAR2CSA regions is optimal. Demonstration that those identified VAR2CSA constructs are the target of naturally acquired cross-reactive and anti-adhesion antibodies should however be of major consideration in the effort to develop an effective VAR2CSA-based vaccine.

In conclusion, genetic immunization by intramuscular plasmid electrotransfer represents a general technology for fast and efficient screening of immunogenic domains within large proteins of which optimal production as recombinant proteins are technically demanding. This work showed that a truncated N-terminal region of VAR2CSA was a major target of anti-adhesion immune response in placental malaria and therefore

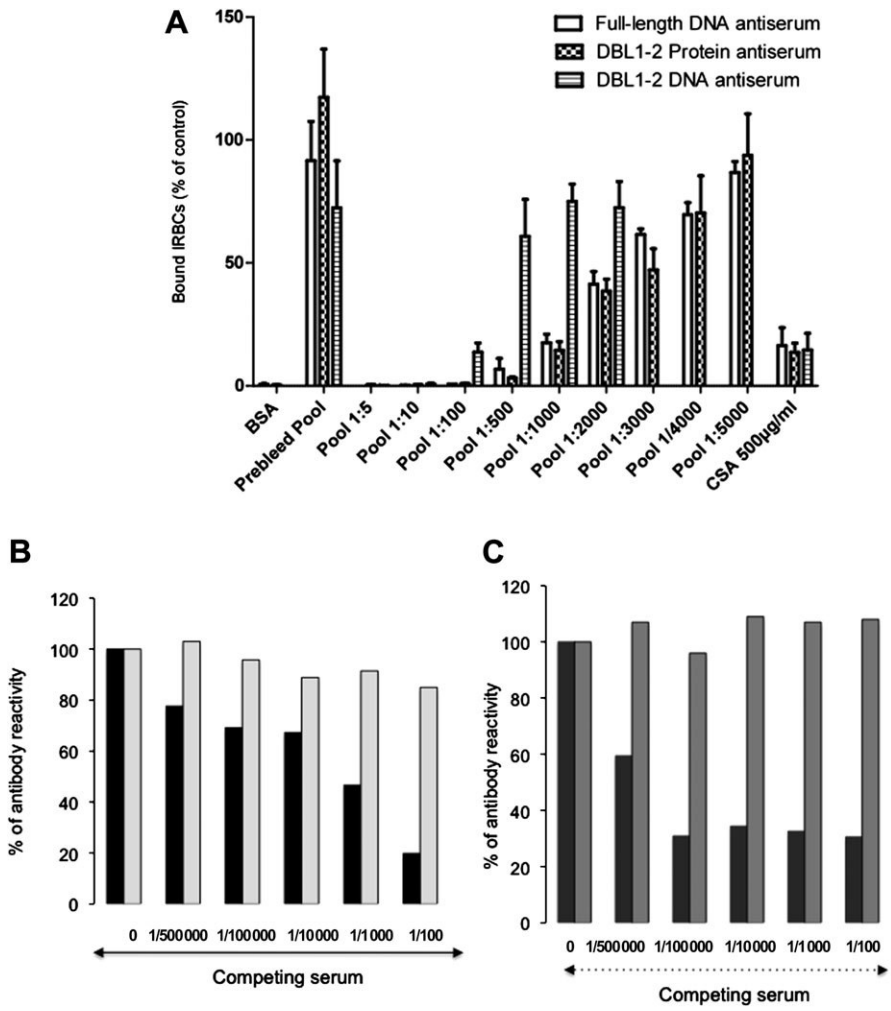

Figure 3. Experimentally induced or naturally acquired antibodies against VAR2CSA-NTS-DBL2X target common epitopes. *A,* Anti-adhesion capacity in a dilution series of hyperimmune mice antisera on the binding of FCR3-BeWo–infected erythrocytes (IEs) to chondroitin sulfate proteoglycan (CSPG). Shown is the proportion of infected red blood cells binding to CSPG in the presence of the indicated dilutions of the D75 antiserum compared with that of control binding without competition. Antisera induced by DNA immunization with the full length (empty histograms) or NTS-DBL2X (stripped histograms) or by the baculovirus-expressed recombinant NTS-DBL2X (dotted histograms) were used. Bovine serum albumin (BSA) indicates the binding of IEs to bovine serum albumin. *B,* Competitive recognition of recombinant NTS-DBL2X between specific antibodies induced by genetic immunization versus protein immunization. Sera from protein-vaccinated mice (■) and the corresponding prebleed (□) are the competing antibodies. D75 serum from DNA vaccinated rabbit is used as noncompeting antibodies. *C,* Competitive recognition of recombinant NTS-DBL2X between antibodies produced by genetic immunization in rabbits and naturally acquired in the plasma of malaria-exposed pregnant women from Benin. The competing sera are: (■) D75 serum from DNA-vaccinated rabbit, and the corresponding rabbit pre-immune serum (■). The noncompeting antibodies are represented by a pool of Beninese multigravidae plasma.

an attractive vaccine target. Further studies are required to ascertain the impact of sequence variation within this particular VAR2CSA region to its potential for cross-reactivity.

Supplementary Data

Supplementary material is available at *The Journal of Infectious Diseases* online.

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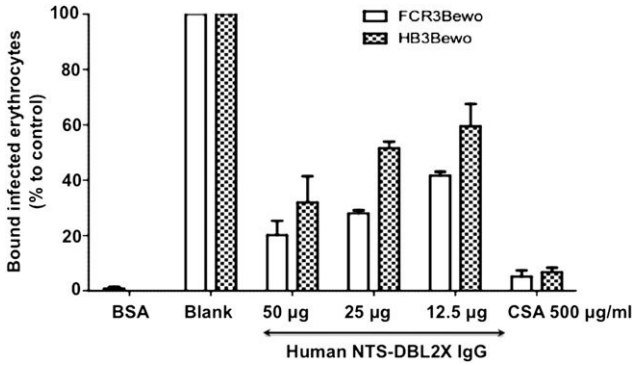

Figure 4. Naturally acquired immunoglobulin G (IgG) against VAR2CSA NTS-DBL2X target strain-transcendent anti-adhesion epitopes. Human IgG specific to NTS-DBL2X was affinity-purified from a pool of plasma from 10 multigravid women that previously showed high anti-adhesion capacities. The FCR3-BeWo and HB3-BeWo infected erythrocytes were incubated with different concentrations (12.5, 25, or 50 $\mu g.mL^{-1}$) of the purified human anti–NTS-DBL2X IgG and the activity was compared with binding without competitor (blank) or soluble competing chondroitin sulfate A (CSA). None of the infected erythrocytes bound to BSA.

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Article IV: Identification of Id1-DBL2X of VAR2CSA as a key domain inducing highly inhibitory and cross-reactive antibodies

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Ce travail s'inscrit dans le même ordre d'idée que le précédent article. La localisation d'un fragment de taille encore plus réduit dans la région d'intérêt de VAR2CSA qui concentre les épitopes protecteurs a incité à la recherche du ou des domaines clés indispensables à une construction fonctionnelle minimale. Allant dans le sens de ces premières observations, plusieurs études ont montré que le site d'adhérence à la CSA, était localisé dans la région N-terminal de VAR2CSA (155) (180).

Les plasmides pVAX1 contenant les constructions NTS-DBL1X, NTS-DBL1X-Id1, Id1, Id1-DBL2X et DBL2X ont été réalisés pour induire des anticorps par vaccination génétique. Les anticorps induit ont été caractérisés par cytométrie en flux pour permettre la reconnaissance de la protéine native et par ELISA pour leur titration et déterminer leur spécificité. Le pouvoir inhibiteur de ces anticorps a été évalué sur des souches de laboratoire sélectionnées sur CSA et sur quelques isolats frais de terrain.

L'analyse du pouvoir inhibiteur de ces anticorps a montré que les constructions NTS-DBL1X, Id1 et DBL2X n'étaient pas capables d'induire des tels anticorps, tandis que la construction NTS-DBL1X-Id1 avait donné lieu à des anticorps dotés d'une activité inhibitrice partielle (40%). En revanche, la construction Id1-DBL2X a permis d'induire des anticorps ayant une forte activité inhibitrice sur l'adhérence des EI à la CSA. Une inhibition similaire à celle observée avec les anticorps dirigés contre le NTS-DBL2X et la partie extracellulaire complète de la protéine. Cette activité inhibitrice du fragment Id1-DBL2X transcende aussi les différentes souches de laboratoire et de terrains utilisées dans cette étude. Il ressort principalement de ce travail que l'inter-domaine Id1 est une composante essentielle dans la construction minimale associant DBL2X.

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Identification of Id1-DBL2X of VAR2CSA as a key domain inducing highly inhibitory and cross-reactive antibodies

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ABSTRACT

Purpose of the research: VAR2CSA is considered as the main target of protective immunity against pregnancy-associated malaria. VAR2CSA high molecular weight complicates scaling up production of VAR2CSA recombinant protein for large-scale vaccination programmes. We previously demonstrated that antibodies induced by NTS-DBL1X-Id1-DBL2X efficiently block parasite binding to CSA in a similar manner to antibodies induced by the full-length extracellular part of VAR2CSA. In order to identifying the shortest fragment of VAR2CSA carrying major protective epitopes able to elicit inhibitory antibodies, we performed a refined antigenic mapping of NTS-DBL1X-Id1-DBL2X through a DNA vaccination technique.
Principal results: Five single or double domains constructs encoding NTS-DBL1X, NTS-DBL1X-Id1, Id1, Id1-DBL2X and DBL2X were made and used to immunize mice. The NTS-DBL1X, NTS-DBL1X-Id1, and Id1-DBL2X fragments all raised high titer immune response, as measured by ELISA. The DBL2X fragment raised a weaker antibody titer, and the Id1 construct failed to elicit antibody. Sera from mice immunized with NTS-DBL1X or DBL2X constructs failed to block infected erythrocytes binding to CSA, whereas sera from mice immunized with NTS-DBL1X-Id1 showed partial inhibitory activity, and the Id1-DBL2X fragment elicited antisera that totally abrogated infected erythrocytes adhesion to CSA. IgG purified from Id1-DBL2X antisera showed a similar inhibitory profile than Id1-DBL2X antisera. Anti-FCR3 anti-Id1-DBL2X antibodies also efficiently block the adhesion of erythrocytes infected by the HB3 parasite line to CSA. Id1-DBL2X antisera recognized the surface of field isolates from pregnant women, and inhibited CSA-binding of all 8 isolates tested, although to a variable level.
Major conclusions: We raised high-titer antibodies against several parts of the protein, and identified Id1-DBL2X as the minimal VAR2CSA fragment inducing antibodies with CSA-binding inhibitory efficiency in the same range as the full-length extracellular part of VAR2CSA.

1. Introduction

Pregnancy-associated malaria (PAM), a major cause of maternal anemia, premature birth and delivery of low birth weight children [1], is characterized by the selective accumulation of *Plasmodium falciparum*-infected erythrocytes (IEs) in placental microvasculature [2]. Tropism and sequestration of IEs to the placenta is mediated through the interaction between a member of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), named VAR2CSA, and chondroitin sulfate A (CSA) on syncytiotrophoblasts [3–5]. VAR2CSA is a large 350 kDa transmembrane protein that includes, in its extracellular region, six Duffy binding like (DBL) domains, three belonging to class X and three to class ε, and four inter-domains (Id) [6].

Numerous lines of evidence support VAR2CSA as a main target of protective immunity against PAM. Parasites in which *var2csa* is genetically disrupted lose their ability to bind to CSA [7,8], suggesting that VAR2CSA is the major PfEMP1 variant associated with binding to CSA. In addition, during first pregnancy, women living in

Abbreviations: PAM, pregnancy-associated malaria; IEs, *P. falciparum*-infected erythrocytes; PfEMP1, *Plasmodium falciparum* erythrocyte membrane protein 1; CSA, chondroitin sulfate A; DBL, Duffy binding like; Id, inter-domains.

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malaria endemic areas are more susceptible to malaria than before being pregnant. This susceptibility is probably due to lack of antibodies against VAR2CSA that is not expressed in parasites infecting non-pregnant hosts, since these pregnancy-specific parasites binds only CSA expressed on the intervillous space in the placenta [9]. Reduced susceptibility to PAM is observed with increasing number of malaria-exposed pregnancies probably owing to the acquisition of specific antibodies [1,10], and multigravid women generally have a higher prevalence and higher titer of anti-VAR2CSA antibodies than primigravid women. Likewise, a high level of anti-VAR2CSA antibodies correlates with protection against the clinical consequences of PAM [11,12]. Moreover, antibodies induced by selected recombinant VAR2CSA domains inhibit the binding of IEs to CSA [13,14]. Recognition of VAR2CSA recombinant domains by sera from donors in malaria-endemic regions is specific to the female gender, correlating directly with gravidity, and transcends the geographic origin of the sera [9,15]. Taken together, these arguments and observations strongly suggest that VAR2CSA carries protective epitopes, and reinforce the possibility of an effective anti-PAM vaccine strategy.

Recently, the full-length extracellular part of VAR2CSA protein has been successfully expressed in eukaryotic systems [16,17], and the inhibitory ability of antisera induced by this protein was tested [16]. Nevertheless, due to VAR2CSA high molecular weight technological constraints still exist, with respect to scaling up production of VAR2CSA recombinant protein as antigen for large-scale vaccination programmes. In addition, the presence of inter-clonal polymorphisms has been a main concern for the feasibility of VAR2CSA-based vaccine since despite the high immunogenicity of full-length VAR2CSA, the induced antibodies are not cross-reactive among various strains or isolates [18]. Thus, the challenge for VAR2CSA-based anti-PAM vaccine development remains the identification of a minimal VAR2CSA region containing conserved protective epitopes that are shared among most VAR2CSA variants in the majority of placental parasite isolates [19].

Although initial studies implicated several distinct DBL domains (DBL2X, DBL3X, DBL5ε and DBL6ε) for CSA binding [20–22], recent data revealed the existence of a single CSA binding site in VAR2CSA recombinant protein, possibly formed by a higher order organization [23]. To explore this key fragment of VAR2CSA comprising the major conserved epitopes responsible for CSA binding, we previously performed antigenic mapping VAR2CSA through DNA vaccination, using a set of overlapping constructs of *var2csa* composed of single or multiple DBL domains [24]. We demonstrated that antibodies induced by NTS-DBL1X-Id1-DBL2X efficiently block parasite binding to CSA in a manner similar to antibodies induced by the full-length extracellular part of VAR2CSA. Other studies are in agreement with our results, indicating the localization of the CSA binding site within the N-terminal domain of VAR2CSA [23,25].

In the present study, in order to reach the ultimate goal of identifying the shortest fragment of VAR2CSA carrying major protective epitopes able to elicit inhibitory antibodies, we have performed a refined antigenic mapping of NTS-DBL1X-Id1-DBL2X through DNA vaccination. The antibodies raised against each fragment were screened for their recognition of the native and recombinant VAR2CSA protein and for their ability to inhibit parasite IEs binding to CSA. Our results indicate that the major epitopes inducing anti-adhesion and, cross-reactive antibodies are located within the Id1-DBL2X fragment. Indeed, Id1-DBL2X (54 kDa) is the shortest region of full-length extracellular VAR2CSA (300 kDa), which is able to induce antibodies that block totally the adhesion of IEs to CSA. Importantly, unlike the antibodies raised against the full-length VAR2CSA, anti-Id1-DBL2X inhibitory antibodies are cross-reactive and, as in such, more compatible with the feasibility of a VAR2CSA-based vaccine strategy.

Table 1
Primers used for PCR amplification of the *var2csa* sub-domains.

Constructs	Primers
NTS-DBL1X	dbl1xF (5′-ACCTCCATAGAAGACACCGGGACCGATCCA-3′)
	dbl1xR (5′-CCGCTCGAGTTAATCACCCTTTATATAATTTTC-3′)
NTS-DBL1X-Id1	dbl1xF (5′-ACCTCCATAGAAGACACCGGGACCGATCCA-3′)
	id1R (5′-CCGCTCGAGTTAATTCGTTAAAGATGCAAATAC-3′)
Id1	id1F (5′-CCACAATTGCCTTATTTCGCAGAATATGC-3′)
	id1R (5′-CCGCTCGAGTTAATTCGTTAAAGATGCAAATAC-3′)
Id1-DBL2X	id1F (5′-CCACAATTGCCTTATTTCGCAGAATATGC-3′)
	dbl2xR (5′-CCGCTCGAGTTAGGCAGCATTTGTAGTACTACTT-3′)
DBL2X	dbl2xF (5′-CCACAATTGAATAAAACATGTATCACACATAGC-3′)
	dbl2xR (5′-CCGCTCGAGTTAGGCAGCATTTGTAGTACTACTT-3′)

2. Material and methods

2.1. Ethics statement

The Beninese women included in this study were given the explanation of the nature of the project and written informed consent was given. The study design, the sampling protocol, as well as the procedure for collecting informed consent, were approved by the "Comité Consultatif de Déontologie et d'Ethique" of the Research Institute for Development (France), and the ethics committee of the Faculty of Health Science (University of Abomey-Calavi, Benin).

Samples from French adult males and pregnant women without previous *P. falciparum* exposure were collected as part of normal treatment procedure, and used as negative controls.

All procedures regarding animal immunizations complied with European and National regulations.

2.2. Plasmid preparation

The *var2csa* gene from FCR3 was used as a cloning template. Gene regions encoding NTS-DBL1X, NTS-DBL1X-Id1, Id1, Id1-DBL2X and DBL2X were amplified by PCR using the oligonucleotide primers as follows (Table 1).

The DNA sequences encoding single or double domains of NTS-DBL1X-Id1-DBL2X were then cloned into a pVAX1 vector backbone (Invitrogen) in which the original cytomegalovirus (CMV) promoter was replaced with the CMV promoter of pCMVβ plasmid (Clontech), and fused to the mEPO secretion sequence as previously described [24,26].

2.3. Animal immunization and antibody collection

In vivo immunizations were carried out on 6-week old Swiss female mice (Janvier-France). Electrotransfer experiments were carried out on mice as previously described [28]. Briefly, mice (5 per group) were anesthetized by intraperitoneal injection of 0.3 ml of a mix of ketamine (100 mg/kg) and xylazine (10 mg/kg) in 150 mM NaCl. Hind legs were shaved and 40 μg of plasmid DNA in saline were injected into the tibial cranial muscle. After injection, transcutaneous electrical pulses (8 pulses of 200 V/cm and 20 ms duration at a frequency of 2 Hz) were applied by two stainless steel external plate electrodes placed about 5 mm apart, at each side of the leg. The mice were immunized three times; at days 0, 30, 60 and antisera were collected 15 days after the last immunizations.

2.4. Antibody reactivity with P. falciparum laboratory lines or field isolates

In vitro propagated *P. falciparum* parasites FCR3 and HB3 were used. Parasites were grown in O^+ erythrocytes without human serum, as described [27]. To select for IEs with antibody recognition characteristics of PAM parasites, FCR3 and HB3 cultures were

repeatedly panned on the human choriocarcinoma cell line BeWo, as described [28]. The derived CSA-adhering IEs, but not the unselected IEs, were recognized in a sex-specific and parity-dependent manner by human plasma groups as described elsewhere [10,29]. We also used *P. falciparum* isolates collected from the placental blood of delivering women at Comé, southwestern Benin, to determine antibody reactivity with primary field isolates. The isolates were maintained *in vitro* for no more than four days before testing.

Flow cytometry (FACS) was used to test the serum reactivity of vaccinated mice to the surface of IEs. Briefly, CSA-selected parasite cultures or field parasite isolates were enriched to contain late trophozoite and schizont stage parasites by exposure to a strong magnetic field (VarioMACS and CS columns, Miltenyi). Aliquots (2×10^5) were labelled by ethidium bromide and sequentially exposed to mouse/human serum and anti-mouse/human IgG-FITC (Invitrogen). Data were acquired using a FACS Calibur flow-cytometer (Beckman Coulter).

2.5. IgG preparation

Total IgG were purified from final bleed mice sera. Briefly, 1 ml of immune serum diluted 1:2 in PBS was manually passed on a Hi-Trap Protein G HP column according to the manufacturer's protocol (GE-Healthcare). Bound IgG were eluted with Tris-Glycin pH 2.4 and dialyzed against PBS. Construct-specific IgG were affinity purified from serum pools of immunized animals using Hi-Trap NHS-activated HP columns (GE Healthcare) on which the NTS-DBL1-2X recombinant protein was coupled following the manufacturer's instructions.

2.6. ELISA assay

To assess antibody responses induced against each construct in vaccinated mice, recombinant protein spanning the NTS-DBL1-2X protein was used in ELISA. The 96-well ELISA plates (Nunc) were coated with 0.5 μg/ml of recombinant protein and incubated at 4 °C overnight. After blocking, diluted plasmas (1/1000) were added to antigen coated wells in duplicate for 1 h at room temperature (RT) followed by a 1/5000 dilution of mouse anti-human IgG for 1 h. Plates were washed and exposed to tetramethyl-benzidine for 10 min, absorbance was read at 450 nm. The IgG serum titers were determined at D75 in all mice sera.

2.7. Inhibition of binding assay for infected erythrocytes

Petri dishes (Falcon) were coated with 20 μl of ligand (1% BSA, 5 μg/ml decorin) diluted in PBS. Spots were incubated overnight at 4 °C in a humid chamber. Each spot was aspirated with a vacuum pump and subsequently blocked with 3% BSA in PBS for 30 min at RT. Late-stage-infected IEs were also blocked in BSA/RPMI for 30 min at RT. Late-stage parasite suspensions adjusted at 20% parasite density were incubated with serum (1:5 final dilution) or purified IgG (0.02 mg/ml to 0.5 mg/ml final concentration) in 1×10^5 cells for 30 min at RT. After removal of the blocking buffer from the spots, 20 μl of antibody/parasite suspension or 500 μg soluble CSA/parasite suspension was quickly added to each spot and incubated for 15 min in a humid chamber at RT. Non-adhering cells were removed by an automated washing system, and spots were fixed with 1.5% glutaraldehyde in PBS for 10 min at RT. The number of adhering IEs was quantified by microscopy and compared to the level of binding in the absence of antibody or soluble CSA. All assays were performed in duplicate and were repeated 2 or 3 times. Only assays performed with field isolates were done only once to avoid the *in vitro* culture of the isolate.

Table 2
Mice sera recognition of VSA expressed on FCR3 IEs after selection by panning on BeWo cells (FCR3-CSA).

Sample	MFI	MFI ratio
Day 0	11.60 ± 1.70	
NTS-DBL1X	40.35 ± 6.43	3.48 ± 0.05
NTS-DBL1X-iD1	47.50 ± 2.40	4.12 ± 0.40
Id1	11.15 ± 1.06	0.98 ± 0.23
Id1-DBL2X	31.75 ± 1.34	2.78 ± 0.52
DBL2X	28.15 ± 2.90	2.43 ± 0.11
NTS-DBL1X-Id1-DBL2X	43.70 ± 7.35	3.76 ± 0.08

At Day 75, reactivity of each immunized mouse serum to the surface of FCR3-CSA IEs was measured by FACS. Data are expressed as the mean fluorescence intensity (MFI), as well as the ratio of D75 mice serum of each construct to pre-immunization (Day 0) serum pool. Results are shown as means ± standard deviations. All tests were done in duplicate. Assays were performed multiple times with similar results.

3. Results

3.1. DNA immunization with NTS-DBL1X-Id1-DBL2X derivative constructs elicits variable antibody titers

Antibodies induced by NTS-DBL1-Id1-DBL2X entirely block IEs binding to CSA of FCR3 strain of *P. falciparum*, as is the case for antisera against full-length extracellular VAR2CSA (NTS-DBL1X to DBL6ε) [24]. Here, we assessed whether the smaller region(s) within the NTS-DBL1X-Id1-DBL2X fragment, contained major epitopes. Five single or double domains constructs encoding NTS-DBL1X, NTS-DBL1X-Id1, Id1, Id1-DBL2X and DBL2X were made in pVax1 vector derivative. Five mice were immunized with each construct through gene-electroporation into skeletal muscle, as described above. The NTS-DBL1X, NTS-DBL1X-Id1, Id1-DBL2X, and DBL2X fragments all raised high titer immune response, as measured by ELISA, comparable to that obtained with NTS-DBL1X-Id1-DBL2X and full-length extracellular part of VAR2CSA (all $p < 0.1$). Although the titer raised by DBL2X seems reduced, high variability in the measurements failed to reach significance. By contrast, the Id1 construct did not elicit any antibody (Fig. 1A). FACS experiments demonstrated that all D75 antisera, but those from Id1-vaccinated mice, recognized the native VAR2CSA protein on the surface of the CSA binding erythrocytes infected with FCR3 parasite line (Table 2).

3.2. CSA-binding inhibitory capacity of the raised polyclonal antibodies

High level immunogenicity and, thereby, a high antibody titer is not necessarily associated with functional importance [14,26]. Thus, in order to evaluate the efficiency of the antisera obtained against NTS-DBL1X, NTS-DBL1X-Id1, Id1-DBL2X and DBL2X, we screened their ability to block the adhesion of erythrocytes infected with VAR2CSA-expressing FCR3 parasites to CSA *in vitro* (Fig. 1B). Sera from mice immunized with either NTS-DBL1X, Id1 or DBL2X constructs failed to block IEs binding to CSA, whereas sera from mice immunized with NTS-DBL1X-Id1 showed partial (60%) inhibitory activity (Fig. 1B). Importantly, vaccination with the Id1-DBL2X fragment elicited antisera that totally abrogated the adhesion of IEs to placental CSA, similarly to antibodies against full length extracellular VAR2CSA and anti-NTS-DBL1X-Id1-DBL2X antibodies (Figs.1B and 2A).

3.3. IgG are involved in the inhibitory activity of Id1-DBL2X antisera

To confirm that the inhibition observed with Id1-DBL2X antisera is IgG-dependent, the IgG from mice immunized by Id1-DBL2X were purified on protein G and tested for their inhibitory activity.

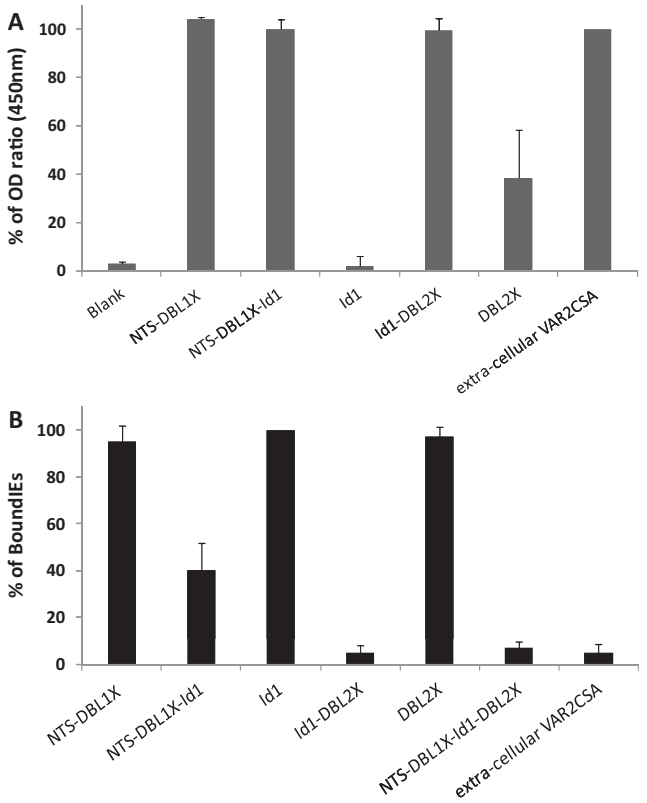

Fig. 1. Antibodies directed against NTS-DBL1X-Id1-DBL2X derivative fragments in immunized mice. (A) Five mice were immunized by each construct. At Day 75 (D75) antibodies induced against each construct were measured by ELISA, with NTS-DBL1X-Id1-DBL2X as capture antigen. Data are expressed as the OD (450 nm) ratio of D75 mice serum pool of each construct to positive controls (NTS-DBL1X-Id1-DBL2X), and each bar is the mean of the antisera obtained from 5 immunized mice, and the line indicates standard deviation. All tests were done in duplicate. Assays were performed multiple times with similar results. Reactivity of sera from mice immunized by NTS-DBL1X, NTS-DBL1X-Id1, Id1-DBL2X, DBL2X, and full length extracellular VAR2CSA did not differ from that with NTS-DBL1X-Id1-DBL2X (all $p > 0.1$). (B) The pool of 5 antisera for each construct was processed in inhibition binding assay with the CSA-selected FCR3 *P. falciparum* strain. Data were expressed as the percentage of bound IEs induced by each pool of immunized mice to mice sera before immunization (D0). Each bar is the mean of the antisera obtained from 5 immunized mice, and the line indicates standard deviation. All tests were done in duplicate. Assays were performed multiple times with similar results. The percentage of bound IEs was significantly reduced (all $p < 0.02$) in the presence of sera from mice immunized by NTS-DBL1X-Id1, Id1-DBL2X, NTS-DBL1X-Id1-DBL2X or full length extracellular VAR2CSA.

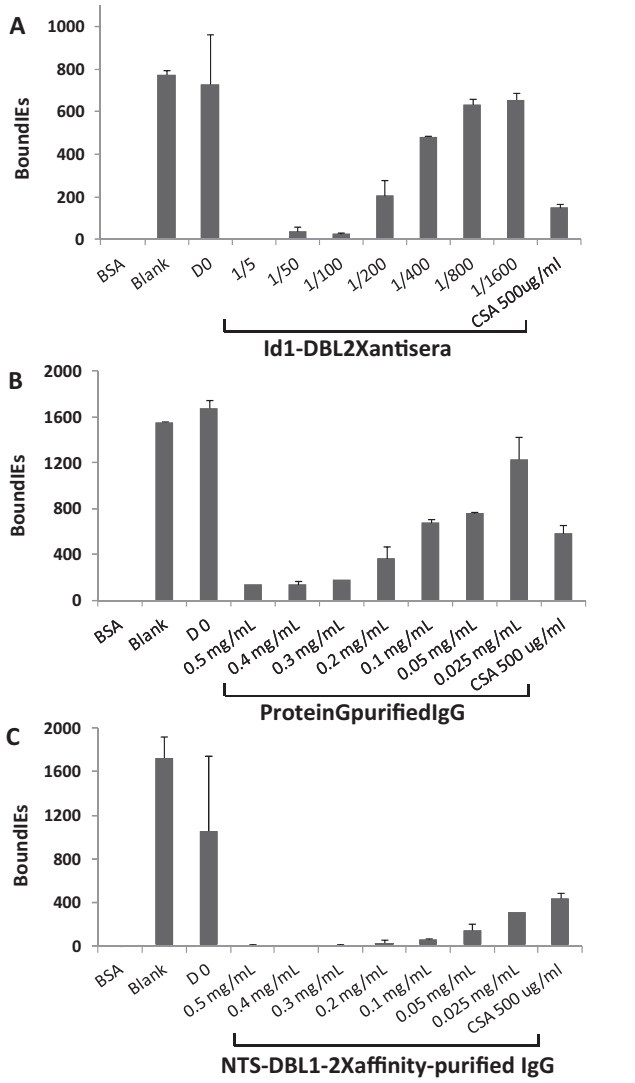

Fig. 2. Inhibition binding assay of mice induced antibodies. (A) The CSA-binding inhibitory capacity of Id1-DBL2X antisera was tested in a range from 1/5 to 1/1600 dilutions, by inhibition binding assay. Blank is medium alone, and CSA is the positive control. The full inhibitory efficiency of Id1-DBL2X antisera is maintained up to 1/200 dilution. Each bar is the mean of the antisera obtained from 5 immunized mice, and the line indicates standard deviation. The number of bound IEs was significantly reduced ($p < 0.05$) in the presence of sera from mice immunized by Id1-DBL2X at all dilutions, except 1/1600. (B) IgG purified from Id1-DBL2X antisera were tested for their inhibitory activity. The purified IgG showed a similar inhibitory profile compared to non purified Id1-DBL2X antisera. Each bar is the mean of the pooled purified IgG from 5 immunized mice, and the line indicates standard deviation. The number of bound IEs was significantly reduced ($p < 0.05$) in the presence of IgG purified form the sera of mice immunized by Id1-DBL2X at all concentrations, excepted 0.025 mg/ml. (C) IgG affinity-purified on NTS-DBL1X-Id1-DBL2X recombinant protein were tested for their inhibitory activity and showed higher efficiency at low concentration. Each bar is the mean of the pooled NTS-DBL1X-Id1-DBL2X specific IgG from 5 immunized mice, and the line indicates standard deviation. The number of bound IEs was significantly reduced ($p < 0.05$) in the presence of IgG purified form the sera of mice immunized by Id1-DBL2X at all concentrations. All tests were done in duplicate. Assays were performed multiple times with similar results.

The purified IgG recognized the surface of selected FCR3 infected IEs and showed a similar inhibitory profile compared to non purified Id1-DBL2X antisera (Fig. 2B). The IgG affinity-purified on NTS-DBL1X-Id1-DBL2X recombinant protein were more efficient and exhibited greater anti-CSA adhesion capacity even at low titers (Fig. 2C).

3.4. CSA-binding inhibitory antibodies induced by Id1-DBL2X are highly cross-reactive

Antibodies induced against the Id1-DBL2X fragment (cloned from *var2csa* of FCR3 parasite line) do efficiently abrogate the CSA-adhesion of erythrocytes infected by the VAR2CSA-expressing FCR3 parasite line. In addition, we investigated the CSA-adhesion inhibitory capacity of these antisera on erythrocytes infected by the HB3 parasite line. Our results demonstrate that anti-FCR3 anti-Id1-DBL2X antibodies also efficiently block the adhesion of erythrocytes infected by the HB3 parasite line to CSA, although a higher concentration of antisera is required (Fig. 3). These indications highlight the cross-inhibitory characteristics of Id1-DBL2X antisera.

3.5. Anti-Id1-DBL2X antibodies specifically recognize isolates from pregnant women and inhibit the adhesion to CSA

Mice Id1-DBL2X antisera were tested by flow cytometric analysis and were shown to specifically recognize the surface of field isolates from pregnant women. Among these isolates, 8 showed specific binding to CSA. The 8 selected isolates were then processed in a binding inhibition assay. The CSA-binding of all the 8 isolates tested was inhibited by specific anti-Id1-DBL2X antibodies (Fig. 4).

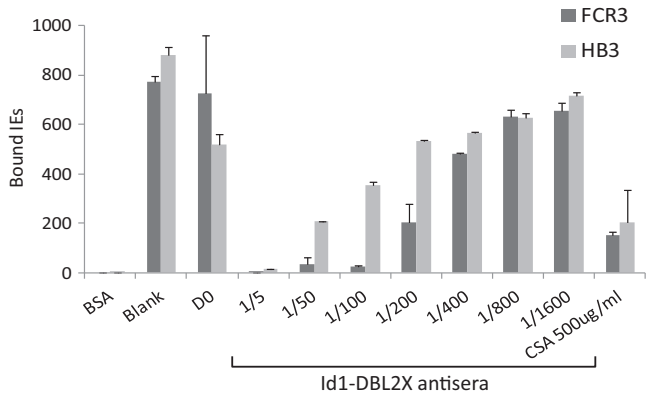

Fig. 3. Mice Id1-DBL2X antisera cross-react with multiple *P. falciparum* lines and inhibit IEs binding. The inhibitory capacity of the mice Id1-DBL2X antisera (in a range of dilutions from 1/5 to 1/1600) was tested on erythrocytes infected by CSA-selected FCR3 and HB3 parasite lines. Antibodies induced by the Id1-DBL2X (cloned from FCR3 *var2csa*) also block the binding to CSA of erythrocytes infected by the HB3 parasite line, although a higher concentration of antisera is required. Each bar is the mean of the antisera obtained from 5 immunized mice, and the line indicates standard deviation. All tests were done in duplicate. Assays were performed multiple times with similar results. The number of bound FCR3 IEs or HB3 IEs was significantly reduced ($p < 0.05$) in the presence of sera from mice immunized by Id1-DBL2X at all dilutions, excepted 1/1600. Up to a 1/400 dilution of antisera, binding inhibition of FCR3 IEs was more potent than that of HB3 IEs; this difference was significant at 1/100 and 1/400 dilutions (both $p < 003$) and borderline at 1/5, 1/50, and 1/200 dilutions (all $p < 0.1$).

The level of inhibition varied with the isolates and reached almost complete inhibition (>90%) in the last two isolates.

4. Discussion

VAR2CSA is considered as the key target for the development of vaccine strategies against PAM since it is the major molecule involved in sequestration of IEs to CSA. Likewise, the main step to reach an effective VAR2CSA-based vaccine consists of the identification of a minimal VAR2CSA region containing conserved protective epitopes that are shared among most VAR2CSA variants expressed by placental parasite isolates.

Several different domains of VAR2CSA, particularly in the N-terminal region, have been implicated in the adhesion to CSA [20,21,24]. We have previously shown that the NTS-DBL1X-Id1-DBL2X region was able to induce antibodies that almost totally abrogate CSA-binding of IEs [24]. In the present paper, we aimed to minimize this fragment as much as possible, while maintaining its CSA-binding inhibitory features, with the intention of reducing technical and financial hurdles and developing a VAR2CSA-based anti-PAM vaccine active against the largest set of *P. falciparum* parasite isolates. In addition, given the diversity of *var2csa* sequences, a central issue for anti-PAM vaccine development is to overcome the existing variability to induce a broad anti-adhesive antibody response. In this study, Id1-DBL2X was the minimal VAR2CSA fragment we identified in which the CSA-binding inhibitory efficiency and specificity was in the same range as that of the full-length extracellular part of VAR2CSA.

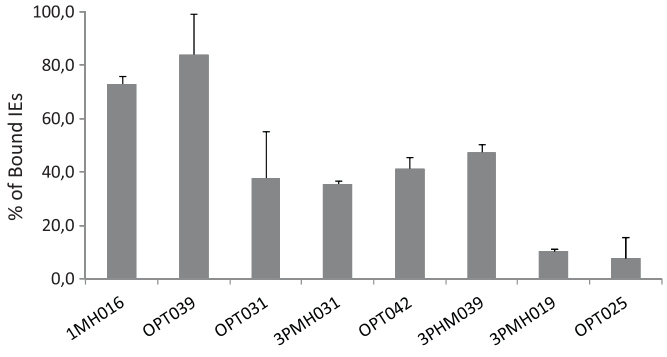

Fig. 4. Inhibition binding assay of mice Id1-DBL2X antisera on field isolates collected in pregnant women from Benin. All 8 isolates that bind specifically to CSA were processed in a binding inhibition assay. Depending to isolates the level of CSA-binding inhibition varied and is illustrated as the percentage of bound IEs in the presence of the pool of mice Id1-DBL2X antisera to negative control (mice antisera on D0). All tests were done in duplicate. Each bar is the mean of the antisera obtained from 5 immunized mice, and the line indicates standard deviation. Sera from mice immunized by Id1-DBL2X significantly reduced ($p < 0.02$) the binding of the 3PHM039 and 3PHM019 isolates. Similar tendency ($p < 0.1$) was observed for isolates 1MH016, 3PMH031, OPT042, and OPT025.

The masking of PfEMP1-specific IgG epitopes by non-specific IgM has been recently documented [30], and may represent a new evasion mechanism allowing placenta-sequestering *P. falciparum* to evade acquired protective immunity without compromising VAR2CSA function or increasing IEs susceptibility to complement mediated lysis. To avoid the effect of IgM, we purified IgG from Id1-DBL2X antisera, and confirmed the inhibitory effect of anti-Id1-DBL2X IgG.

Interestingly, as compared to Id1-DBL2X antisera that completely inhibit IEs adhesion, the NTS-DBL1X-Id1 construct induces antibodies with a partial (up to 40%) inhibitory activity, while neither NTS-DBL1X nor DBL2X on their own induce inhibitory antibodies, and Id1 alone elicits no antibody response at all. Nevertheless, as the data presented here clearly reveal, the expression in tandem with full-length Id1 is a fundamental necessity for the induction of adhesion-inhibitory antibodies by either NTS-DBL1X or DBL2X. These data suggest that Id1 is an integral component of the epitope, putatively conformational, that induces antibodies that block IEs adhesion to the placenta.

Epitope mapping in this study is performed by using a DNA immunization approach, since *in vivo* expression ensures that the protein bears closer similarity to the normal eukaryotic structure and configuration, with accompanying post-translational modifications and conformational folding. Indeed, it was recently shown that anti-NTS-DBL1X-Id1-DBL2X antibodies elicited through DNA-immunization faithfully maintain the properties of the same antibodies induced by protein immunization [24]. Moreover, DNA immunization is faster than protein immunization, avoiding the need for recombinant protein production, itself a long costly and limiting step.

In the present paper, we have raised high-titer antibodies against several parts of the protein, and have identified Id1-DBL2X in the N-terminal part of the VAR2CSA as an epitope inducing highly inhibitory antibodies. The fact that the adhesion to CSA of various *P. falciparum* isolates is inhibited to a variable level may reflect differences in sequences due to the high level of polymorphism of the VAR2CSA gene. Indeed, a combination of several Id1-DBL2X might be required to induce a cocktail of broad inhibitory antibodies able to completely abrogate CSA-binding of all isolates from pregnant women.

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Article V: Differential adhesion-inhibitory patterns of antibodies raised against two major variants of the NTS-DBL2X region of VAR2CSA

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A la suite des travaux d'identification de la région minimale d'intérêt dans la protéine VAR2CSA, et bien que les anticorps dirigés contre cette région fussent capables de bloquer l'adhérence des EI à la CSA de plusieurs isolats de parasites infectant les femmes enceintes, cette activité inhibitrice n'a pas d'effet sur certains isolats de patients. Les travaux de Sander en 2009, qui ont permis de mettre en évidence un dimorphisme de séquence antigénique dans le DBL2X ont servi d'hypothèse à cette nouvelle étude. Cette hypothèse vise la complémentarité fonctionnelle des anticorps induits contre les représentants des deux sous-familles de séquences. Le motif de séquence dimorphique de 36 acides aminées, caractérisant deux groupes distincts d'isolats contenant chacun les variants FCR3 et 3D7. Ce travail a eu pour objectif d'évaluer les propriétés anti-adhérence des anticorps dirigés contre la construction NTS-DBL2X extraite à partir des variants FCR3 et 3D7.

Le typage moléculaire de 122 isolats de femmes enceintes par PCR-RFLP, développé pour cibler ce motif de séquence dimorphique, a permis de mettre en évidence une distribution similaire de deux signatures moléculaires dans notre échantillonnage. Toutefois, chez 17% des isolats analysés, la présence des deux motifs était observée traduisant ainsi la polyclonalité des infections qui peuvent contenir les deux variants antigéniques à la fois. Le niveau de marquage de la protéine native à la surface des EI a été évalué sur 47 isolats et a montré que les anticorps dirigés contre les deux constructions du NTS-DBL2X peuvent marquer la protéine native VAR2CSA exprimée par ces isolats de façon similaire et indépendamment de leur fond génétique. La capacité anti-adhésion de ces anticorps a été évaluée sur 18 isolats à fond génétique variable. Cependant, bien que les anticorps anti-NTS-DBL2X des souches FCR3 et 3D7 soient capables d'inhiber l'adhérence de plusieurs isolats au CSPG, l'analyse combinée de l'activité inhibitrice de ces anticorps a permis de démontrer

une complémentarité d'inhibition. Les isolats échappant à un anticorps sont le plus souvent, inhibés par l'autre préparation. Ce chevauchement d'activité sur les isolats de femmes enceintes a montré une possibilité d'inhiber la totalité des isolats testés dans cette étude.

Ce travail est le premier à avoir mis en évidence, une activité souche-dépendante des anticorps induite contre des constructions de VAR2CSA. Cette activité a été démontrée sur les lignées 3D7 et FCR3 dont les anticorps induits sont plus actifs sur le parasite homologue. La mise en évidence d'une possible optimisation de l'activité des anticorps anti-VAR2CSA sur les isolats de terrain, par la combinaison d'au moins deux variants de la protéine, pourraient constituer un aspect majeur à prendre en compte dans le développement d'un vaccin efficace à base de VAR2CSA.

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Differential adhesion-inhibitory patterns of antibodies raised against two major variants of the NTS-DBL2X region of VAR2CSA

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ABSTRACT

Background: VAR2CSA is a large polymorphic Plasmodium falciparum protein expressed on infected erythrocytes (IE) that allows their binding in the placenta, thus precipitating placental malaria (PM). The N-terminal part of VAR2CSA that contains the binding site to placental chondroitin sulfate A (CSA) is currently recognized as the most attractive region for vaccine development. An ultimate challenge is to define epitopes in this region that induce a broad cross-reactive adhesion inhibitory antibody response.
Methods: Based on phylogenetic data that identified a dimorphic sequence motif in the VAR2CSA DBL2X, we raised antibodies against the NTS-DBL2X constructs containing one sequence or the other (3D7 and FCR3) and tested their functional properties on P. falciparum isolates from pregnant women and on laboratory-adapted strains.
Results: The CSA binding inhibitory capacity of the antibodies induced varied from one parasite isolate to another (range, 10%–100%), but the combined analysis of individual activity highlighted a broader functionality that increased the total number of isolates inhibited. Interestingly, the differential inhibitory effect of the antibodies observed on field isolates resulted in significant inhibition of all field isolates tested, suggesting that optimal inhibitory spectrum on field isolates from pregnant women might be achieved with antibodies targeting limited variants of the N-terminal VAR2CSA.
Conclusions: Our findings indicate that the NTS-DBL2X region of VAR2CSA can elicit strain-transcending anti-adhesion antibodies and suggest that the combination of the two major variants used here could represent the basis for an effective bivalent VAR2CSA-based vaccine.

1. Introduction

The pathogenesis of malaria during pregnancy results from the selective accumulation of *Plasmodium falciparum* infected erythrocytes (IE) in the intervillous spaces of the placenta [1,2]. This placental sequestration of IE severely increases the risks of miscarriage, maternal anemia and low birth weight [3]. A member of the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) family, named VAR2CSA, is the parasite ligand for chondroitin sulfate A (CSA) [4], a glycosaminoglycan present in the placenta. CSA is considered to be the principal or only placental receptor for IE [5], and selective disruption of the *var2csa* gene abrogates the binding ability of IE to CSA *in vitro* [6]. VAR2CSA has been shown to be predominantly expressed by placental isolates [7–9], and plasma from individuals in malaria-endemic regions recognizes VAR2CSA-expressing isolates in a sex-specific and parity-dependent manner [10,11]. High plasma levels of anti-VAR2CSA antibodies are associated with improved pregnancy outcomes in areas where women are exposed to *P. falciparum* infections [10–12]. Recombinant proteins reproducing selected extra-cellular domains of VAR2CSA

Abbreviations: PM, placental malaria; IE, Pfalciparum-infected erythrocytes; *Pf*EMP1, *Plasmodium falciparum* erythrocyte membrane protein 1; CSA, chondroitin sulfate A; CSPG, chondroitin sulfate proteoglycan; DBL, Duffy binding-like; Id, inter-domain; NTS, N-terminal sequence; DSM, dimorphic sequence motif; PBS, phosphate buffered saline; BSA, bovine serum albumin; MFI, median fluorescence intensity; IPTp, intermittent preventive treatment during pregnancy.

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are able to induce antibodies that efficiently inhibit CSA-binding of IE [13–16]. The combination of these observations clearly identifies VAR2CSA as the main target of acquired protective immunity to placental malaria (PM), and it is thus a candidate for the development of a vaccine against this clinical malaria syndrome.

VAR2CSA is a large protein with a molecular weight of ~350 kDa formed by six Duffy binding-like domains (DBL1-6) and four inter-domains (Id1-4) [17–19]. One of the major challenges in vaccine development is to identify a minimal region that induces strain-transcending inhibitory antibodies. We recently showed that antibodies to the N-terminal region of VAR2CSA display broad and strong adhesion-blocking capacity on field isolates [20,21]. The identification of the minimal CSA-binding in this region [22–24], suggests that efficient anti-adhesion and very likely naturally-acquired protective antibodies bind to this part of VAR2CSA.

Another challenge to vaccine development is the sequence polymorphism present in VAR2CSA [8,18]. Antibodies raised against a single FCR3 variant of NTS-DBL2X inhibited CSA-binding of field isolates with an average inhibition level of 65% (range, 32–95%) [20], suggesting that antibodies against more than one variant would be needed for optimal protection. We have previously identified a dimorphic sequence motif (DSM) in the DBL2X domain of VAR2CSA [25] with two distinct phylogenetic groups of FCR3 and 3D7 types. In this study, we examined the prevalence of each DSM type among *P. falciparum* parasites isolated from Beninese pregnant women. Furthermore, we examined the adhesion inhibitory capacity of antibodies raised against each of the NTS-DBL2X variants on field and laboratory-maintained isolates.

2. Materials and methods

2.1. Ethics statement

Pregnant women were included in this study after written informed consent was obtained. The study was approved by the ethical committee of the Faculty of Health Science (University of Abomey-Calavi, Benin). Animal immunization followed the FELASA (Federation of Laboratory Animal Science Associations) guidelines and was approved by the ethical committee affiliated to the University Paris Descartes.

2.2. P. falciparum isolates

P. falciparum IE were obtained from pregnant women at Suru Léré maternity clinic, Cotonou, Benin. The study site is characterized by hyper-endemic malaria and high transmission with two peaks during the two rainy seasons [26]. Pregnant women attending antenatal visit or admitted for delivery were screened for malaria using a rapid diagnostic test Parascreen™ (Zephyr Biomedicals Goa, India). Eight milliliters of venous blood were collected from women with plasmodial infection, in vacutainers with citrate phosphate dextrose adenine anticoagulant. Two hundred μl of erythrocyte pellets were stored at −20 °C for subsequent DNA extraction or homogenized in 10 volumes of TRIzol reagent (Invitrogen) and stored at −80 °C until RNA extraction. IE pellets were immediately cultured *in vitro* to trophozoite-stage as described [27]. Briefly, isolates were grown in RPMI 1640 supplemented with Hepes and L-glutamine (Lonza Biowhittaker), 0.3 g/L L-glutamine, 0.05 g/L gentamicin, 5 g/L albumax. Cultures were grown for no more than 48 h before testing. Laboratory-adapted parasite strains FCR3, HB3 and NF54 were also grown and selected following several panning on the choriocarcinoma cell line BeWo, as described [28].

2.3. DNA extraction and msp genotyping

DNA was extracted from 100 μl of blood pellet using GeneJet Genomic Purification Kit (Fermentas) as recommended by the manufacturer. *Msp1* and *msp2* genes were amplified by nested PCR using specific primers [29]. Multiplicity of infection (MOI) was determined as the average number of distinct fragments detected in each sample.

2.4. RNA extraction, cDNA synthesis and DBL2X genotyping

Total RNA was extracted from samples stored in TRIzol reagent (Invitrogen), as recommended by the manufacturer. To remove potential contamination by genomic DNA (gDNA), RNA samples were treated with DNase I (Invitrogen) for 15 min at room temperature (RT). Absence of gDNA in RNA samples was confirmed by no amplification in real-time PCR using primers targeting the housekeeping gene *seryl-tRNA synthetase* [4]. Reverse transcription was performed using Thermoscript (Invitrogen) with random hexamer primers, as recommended.

To type the previously described DSM in the VAR2CSA DBL2X domain of *P. falciparum* isolates [25], the DBL2X gene sequence was selectively amplified from cDNA of each isolate using high fidelity Fusion Taq Polymerase. Primers forward 5′-TTAYCCCC-AAGAACACA-3′ and reverse 5′-TTTTAAATTTTTTCCATGAA-3′ were used under the cycling conditions 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 68 °C for 50 s, with final extension at 68 °C for 10 min. PCR products were digested with restriction enzymes *BstCI* (cuts the FCR3 DSM-type) and *Hppy188I* (cuts the 3D7 DSM-type) for 1 h at 50 °C and 37 °C, respectively.

2.5. Antibody production and IgG preparation

The NTS-DBL2X fragments and the full-length sequence of the optimized *var2csa* gene from the FCR3 and 3D7 parasite lines were used to produce specific anti-VAR2CSA IgG, by DNA vaccination as described [20]. Briefly, DNA sequences were cloned into a pVax1 vector derivative and fused to the mEPO signal sequence as described [30]. Anaesthetized New-Zealand rabbits (Janvier-France) were immunized by injection followed by application of transcutaneous electric pulses in 5 sites of each *longissimus dorsi* muscle. Animals were immunized at days 0, 30 and 60, and antisera were collected at day 0 and 75 days. Total IgG were purified from non-immunized (day 0) and immune rabbit (day 75) sera on a Hi-Trap Protein G HP column, according to the manufacturer's recommendations (GE-Healthcare).

3. Flow cytometry assay

The reactivity of IgGs to the surface of *P. falciparum* IEs was analyzed by flow cytometry (FACS Calibur) as described [31]. Briefly, CSA-adhering lines (FCR3-Bewo and HB3-Bewo) and field parasite isolates were enriched by exposure to a strong magnetic field (VarioMACS and CS columns, Miltenyi). For each test 2×10^5 IE were labeled with ethidium bromide, sequentially exposed to rabbit IgG (final concentration approximately 10 μg/ml) for 30 min, and to FITC-conjugated anti-rabbit for 30 min (Invitrogen). Data were acquired, analyzed and the median fluorescence intensity (MFI) was determined. Labeling was defined positive with MFI ratio >1.2 (MFI with post-immunization IgG at day 75/MFI with pre-immunization IgG (negative control at day 0), as described [31].

3.1. Inhibition of binding assay (IBA)

Antibodies' capacity to inhibit IE binding to chondroitin sulfate proteoglycan (CSPG) was explored using a static Petri dish assay, as

Table 1
Characteristics of the study population.

Characteristics	All women ($n=123$)	Samples in Flow analysis ($n=47$)	Samples selected for IBA[b] ($n=18$)
Age, years median (IQR[a])	24 (20–30)	25 (21–30)	24 (21–30.5)
Median of parity (IQR)	2 (1–4)	2 (1–4)	2.5 (1–4)
Gestational Age, median (IQR)	20 (15–28)	23 (12–29)	25.5 (17.5–31.3)
MOI, median (IQR)	3 (2–4)	3 (1–4)	4 (2–4.5)
Parasitemia (IQR), parasites/μl	1638.5 (311.5–18,177.5)	11340 (2937.3–54,996.5)	23,200 (3049–81300)

[a] Interquartile range.
[b] Inhibition binding assay.

described [20]. Briefly, 20 μl of 5 μg/ml CSPG-Decorin (Sigma) or 10 μg/ml bovine serum albumin (BSA) diluted in PBS were coated as spots in a 100 mm × 15 mm Petri dish (Falcon 351029). Spots were incubated overnight at 4 °C in a humid chamber and blocked with 3% BSA. Late-stage IE with a parasitemia adjusted to 20% in 1×10^5 cells were incubated with purified IgG (250 μg/ml) or 500 μg/ml of soluble CSA (Sigma). Cells were allowed to bind to coated-plates for 15 min at RT. Non-adherent cells were removed by an automated washing system. Spots were fixed with 1.5% glutaraldehyde, stained with Giemsa and adherents IE were quantified by microscopy.

4. Statistical analysis

All experiments were performed in duplicate. Kruskall–Wallis, Mann–Whitney or Wilcoxon rank sum tests were used to compare continuous variables while categorical variables were compared using the Fisher exact test. Prism software (version 4; Graphpad) was used to plot data and statistical analyses were performed by using STATA 12 software. Significance was defined at P values ≤ 0.05.

5. Results

5.1. Determination of multiplicity of infection and molecular typing of var2csa among parasites isolated from pregnant women

A total of 1538 pregnant women were screened by RDT, from May through August 2012. Active *P. falciparum* infection was confirmed by microscopy on 123 women (8% prevalence). Characteristics of the women enrolled in the study are presented in Table 1. The mean MOI of these isolates was 3.1 (range, 1–7). cDNA was obtained from 122 isolates. A PCR–RFLP was performed on all field isolates to investigate the distribution of the DBL2X DSM. A restriction enzyme-based analysis of this dimorphic region revealed the presence of at least one of the dimorphic variants in transcripts from all parasites [25]. Fifty-four isolates (44%) transcribed only the 3D7 DSM-type while the FCR3 DSM-type was the only genotype detected in the transcripts of 47 isolates (39%). Both types of transcripts were detected in 21 isolates (17%), suggesting a mixture of genotypes (Fig. 1).

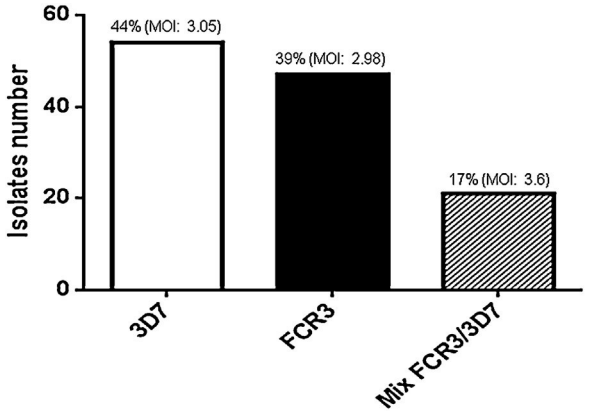

Fig. 1. Distribution of the dimorphic sequence signatures of VAR2CSA DBL2X among parasites infecting pregnant women. The dimorphic sequence motif (DSM) in DBL2X that discriminates the *var2csa* alleles into two subgroups (the 3D7 and FCR3 types) was genotyped in parasites collected from pregnant women in Benin. Shown are proportions of 3D7 type (white histogram), FRC3 type (black histogram) and the mixture of both genotypes (hashed histogram). The multiplicity of infection (MOI) was indicated for each category.

5.2. Specific IgG induced against the NTS-DBL2X of FCR3 and 3D7 types recognized isolates from pregnant women equally well

The labeling of VAR2CSA expressing IE was measured by flow cytometry on 47 freshly isolated, *in vitro* matured *P. falciparum*-IE. Among these isolates, 16 transcribed the 3D7 DSM, 21 the FCR3 DSM and both genotypes were detected in 10 isolates. Both anti-NTS-DBL2X IgG against the 3D7 and FCR3 variants, and IgG raised against the full-length VAR2CSA were used in this assay. IgG purified from non-immunized rabbit showed no significant reactivity with all tested isolates. The range of MFI values are shown in the supplementary Table 1. Higher levels of reactivity were observed with antibodies against the full-length VAR2CSA (median = 1.4) as compared to antibodies directed to the NTS-DBL2X fragments (medians, 1.13 and 1.17 for 3D7 and FCR3 respectively) ($P < 0.05$). No difference was found in the reactivity of the two types of anti-NTS-DBL2X IgG ($P = 0.59$). This reactivity was similar on isolates expressing either the 3D7 or FCR3 DSM-type, (Fig. 2, $P = 0.66$ for NTS-DBL2X/FCR3 and $P = 0.63$ for NTS-DBL2X/3D7).

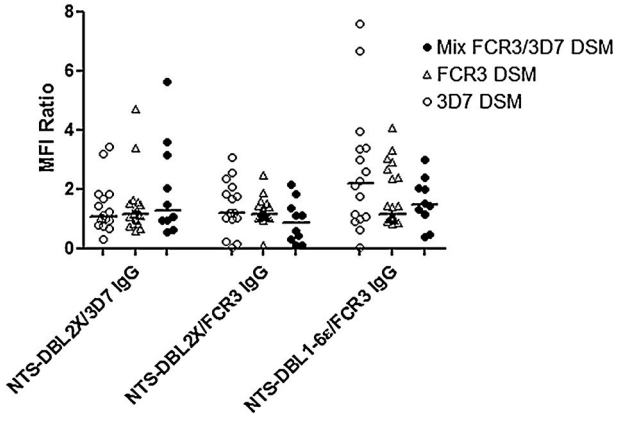

Fig. 2. Recognition of surface expressed VAR2CSA in field isolates by IgG raised against VAR2CSA constructs. By flow cytometry analysis, the expressed VAR2CSA recognition ability of IgG elicited to NTS-DBL2X constructs (NTS-DBL2X/3D7 and NTS-DBL2X/FCR3) and to the full-length extra-cellular part of VAR2CSA of the FCR3 strain (NTS-DBL1-6ε/FCR3) was assessed on field isolates. Parasites were segregated according to their DSM category (3D7, FCR3 or Mix FCR3/3D7 DSM). Bar indicates the median of the MFI ratio (MFI corresponding to the reactivity of IgG from hyper-immune animal/MFI corresponding to the reactivity of pre-bleed IgG).

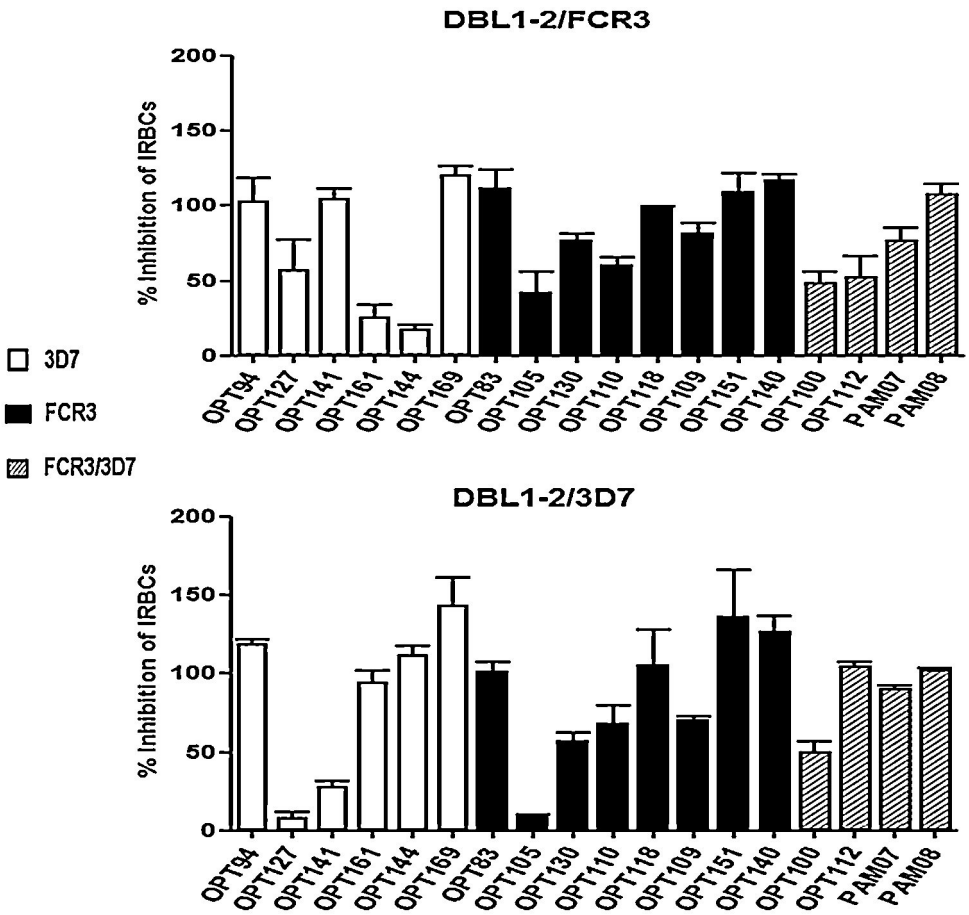

Fig. 3. Adhesion inhibitory activity of IgG raised against each of the two variants of NTS-DBL2X on field isolates. Binding inhibitory activity was evaluated on 18 freshly collected *P. falciparum* isolates from pregnant women. Data are shown as percent of inhibition of each antibody that were normalized by CSA inhibition value (used here as a reference of the maximal binding inhibition). An arbitrary threshold of two times the limit observed on a panel of pre-bleed rabbit sera was defined at 40% inhibition. Histograms were labeled according to DSM type detected in the isolate: (□) for 3D7 DSM type, (■) for FCR3 DSM type (▨) for mixture of both genotypes.

5.3. Adhesion inhibitory capacity of anti-NTS-DBL2X IgGs

The anti-adhesion capacity of the antibodies was further assessed on 18 isolates collected from pregnant women in Benin. Weakly binding isolates (<250 IE/mm^2) were excluded from this testing. All of the 18 highly binders tested in the IBA were significantly labeled by anti-VAR2CSA IgGs while excluded isolates were weakly or not recognized (all $P < 0.05$). No difference was observed between excluded and included isolates regarding the clinical characteristics of parasite donors or the DSM distribution. Six of the highly binding isolates expressed only the 3D7 DSM-type, 8 had only the FCR3 DSM-type, and a mixture of both types was found in 4. Significant adhesion inhibition was obtained with post-immunization IgG induced with both NTS-DBL2X constructs (Table 2). Data were further normalized against that of soluble CSA used as reference of the maximal binding inhibition as presented in Fig. 3. The interquartile range (IQR) of inhibitory activity of post-immunization antibodies on all isolates was [51–100%] for anti-NTS-DBL2X/FCR3 IgG and [55–100%] for anti-NTS-DBL2X/3D7. No inhibition was observed with pre-immunization IgG. Sixteen out of the 18 isolates tested were significantly inhibited (range, 43–100%) by anti-NTS-DBL2X/FCR3 IgG, whilst a high inhibitory activity of anti-NTSDBL2X/3D7 was observed on 15 out of 18 isolates tested (range, 50–100%). No significant difference was observed overall ($P = 0.84$) in the activity of both types of antibodies according to the DSM expressed by the isolates. However, significantly contrasting activity of both antibodies can be observed on the same parasite (Fig. 3). Of the 8 isolates expressing only the FCR3-like DSM, binding to CSPG for all was significantly blocked by anti-NTS-DBL2X/FCR3 (range, 43–100%) while binding of all but one isolate was inhibited by antibodies against the 3D7 type. Likewise, of the 6 isolates transcribing the 3D7 DSM-type, only 4 were actually inhibited by antibodies against each DSM-type, and 2 different isolates were not inhibited. Isolates OPT161 and OPT144 expressing the 3D7 DSM-type, were inhibited by IgG specific to the NTS-DBL2X/3D7 variant, but not by antibodies raised against the FCR3 variant. The opposite profile was observed with isolates OPT127 and OPT141 expressing the 3D7 DSM-type, which were inhibited by IgG specific to the FCR3 DSM-type and not by IgG against the homologous DSM.

The two IgG types were further tested individually or as a mixture on laboratory-adapted parasite lines selected on BeWo cells. The FCR3 and HB3 lines (which share the FCR3 DSM-type), and the NF54 (3D7 background) were used. High inhibitory activity was consistently obtained with antibodies toward IE belonging to homologous DSM. Anti-NTS-DBL2X/FCR3 IgGs completely inhibited CSA-binding of FCR3 and HB3 parasite lines (Fig. 4), but had no effect on binding of the NF54 parasite line. Anti-NTS-DBL2X/3D7 IgG highly inhibited (85%) the CSA-binding of NF54 parasite, and had only partial effect (20–40%) on the FCR3 and HB3 parasite lines. Interestingly, a mixture of anti-NTS-DBL2X IgG from both FCR3 and 3D7 strains conserved the ability to almost totally abrogate (>82%) the binding of all 3 parasite lines to CSPG.

Table 2
Adhesion inhibitory capacity of antibodies induced against two variants of the NTS-DBL2X constructs on *P. falciparum* infected erythrocytes collected from pregnant women.

Isolates	Gestational age of women	DSM-type	MFI Ratio for NTS-DBL2X/3D7	MFI Ratio for NTS-DBL2X/FCR3	MFI Ratio for NTS-DBL6ε/FCR3	Bound IE/mm2 on BSA	Bound IE/mm2 on CSPG	NTS-DBL2X/3D7 inhibition (%)		NTS-DBL2X/FCR3 inhibition (%)		CSA inhibition (%)
								Prebleed	Immune	Prebleed	Immune	
OPT094	12	3D7	1.1	1.2	3.0	3	417	23.00	91.59	15.27	73.05	76.96
OPT127	31	3D7	1.0	1.7	3.4	1	446	22.45	7.74	5.78	49.06	82.14
OPT141	24	3D7	1.0	2.1	3.7	1	402	4.28	22.88	20.00	86.25	82.00
OPT161	18	3D7	1.2	0.9	1	1	401	5.38	73.98	8.57	20.49	77.47
OPT144	28	3D7	1.7	1.1	1.7	1	414	6.15	89.74	0.00	14.96	80.05
OPT169	16	3D7	1.8	3.1	6.7	2	263	0.00	89.15	1.38	75.80	62.45
OPT083	22	FCR3	1.5	1.4	1.2	1	274	0.00	88.08	0.00	97.07	87.21
OPT105	38	FCR3	1.4	1.3	2.7	3	255	8.15	6.98	12.81	31.24	79.19
OPT109	27	FCR3	1.1	2.5	2.3	2	275	10.00	63.70	13.42	73.23	89.63
OPT110	37	FCR3	1.1	1.4	1.4	2	261	17.00	50.45	17.32	44.61	75.50
OPT118	29	FCR3	1.3	1.5	3.6	3	250	0.00	73.19	10.00	70.16	70.81
OPT130	24	FCR3	1.2	1.9	4.0	2	746	9.45	48.12	15.72	65.04	84.31
OPT140	33	FCR3	1.3	1.3	1.6	3	253	16.72	96.36	15.40	89.56	76.18
OPT151	28	FCR3	4.7	1.6	3.0	2	715	0.00	91.10	0.00	73.98	69.02
OPT100	32	Mix	1.0	1.1	1.3	3	552	11.54	42.77	0.00	47.86	90.05
OPT112	20	Mix	1.1	1.3	3.0	4	244	0.00	94.36	1.14	47.75	90.00
PAM007	37	Mix	3.1	1.8	3.1	1	1256	0.42	72.45	21.00	61.32	80.00
PAM008	39	Mix	3.6	2.2	2.9	0	964	10.51	93.62	0.00	98.60	91.38

Inhibition of binding was calculated as percent relative to blank (binding without competitor).

6. Discussion

Several lines of evidence identify VAR2CSA as the key target for the development of a vaccine to prevent PM [4,7,8,32]. However, constraints that include the high molecular weight of VAR2CSA and inter-clonal polymorphisms are the major obstacles in the process of vaccine development. An effective vaccine should elicit broadly reactive antibodies that inhibit adhesion of IE to placenta regardless of primary sequence variations between isolates. An optimal region within VAR2CSA that retains functional and strain-transcending epitopes was previously identified in the NTS-DBL2X fragment [20,21]. As some isolates remained weakly or not inhibited by antibodies raised against a single variant of NTS-DBL2X construct from the FCR3 parasite strain, this invited further analysis to understand the contribution of antigenic polymorphism to the adhesion inhibitory capacity of anti-VAR2CSA antibodies. In this study we were interested in the DBL2X where a site with dimorphic polymorphism was previously described. Although this VAR2CSA domain taken alone does not bind CSPG, it was essential for optimal binding ability of a bigger construct and induction of anti-adhesion antibodies [21,24]. The DSM defined in DBL2X can discriminate two major variants (FCR3 and 3D7) and the existence of this clear-cut dichotomy motivated the exploration of the ability of antigens bearing these signatures of inducing antibodies with similar properties. We first investigated the distribution of the two DSM variants among *P. falciparum* parasites isolated from pregnant women in Benin, and assessed the capacity of antibodies generated against two DSM-specific NTS-DBL2X constructs to alter IE adhesion to CSPG. The antibodies raised against NTS-DBL2X/FCR3 or NTS-DBL2X/3D7 variants were able to label the surface of several isolates at a similar magnitude, irrespective of the DSM type expressed. Higher level of labeling, however, was noted with antibodies raised against the full-length construct of VAR2CSA. This might be explained by the fact that surface recognition involves several epitopes on several domains, some of which might be immuno-dominant and will increase the labeling ability of antibodies to a larger protein than truncated ones. Interestingly, antibodies raised against each NTS-DBL2X construct could block the binding to CSPG of several field isolates, suggesting that these antibodies target strain-transcendent epitopes supporting IE adhesion. The evaluation of the antibodies on the phenotypically well-characterized laboratory parasite strains clearly highlighted distinct inhibitory properties on the homologous and heterologous strains as defined on the genetic background relative to the DSM. This differential activity is also visible on field isolates, where one can easily note distinct patterns of inhibition by the two kinds of antibodies on 5 of the 18 isolates analyzed. Although the DBL2X dimorphic antigenic polymorphism does not seem to totally explain the variation in the inhibitory capacity of the antibodies on various field isolates, as there is no significant difference in our data set to suggest that binding inhibitory antibodies are DSM specific, it is still striking to note that the adhesion of those 5 field isolates which was not inhibited by antibodies with specificity for one construct was indeed inhibited by antibodies with specificity for the other construct. The combination of the overlapping inhibitory patterns of both types of antibodies analyzed here and showing differential inhibitory properties on some isolates actually resulted in significant inhibition of all isolates studied. We assume that the few critical and functional variations between isolates are covered by the two different antigenic constructs. This observation is of major importance in the ongoing effort to create an optimal VAR2CSA-based vaccine that will likely be essential for the induction of a sufficiently protective immunity. The data from this study, although generated on a small number of samples, clearly suggest that a higher proportion of parasite isolates would be targeted by a multivalent vaccine approach comprising of two genotypes or

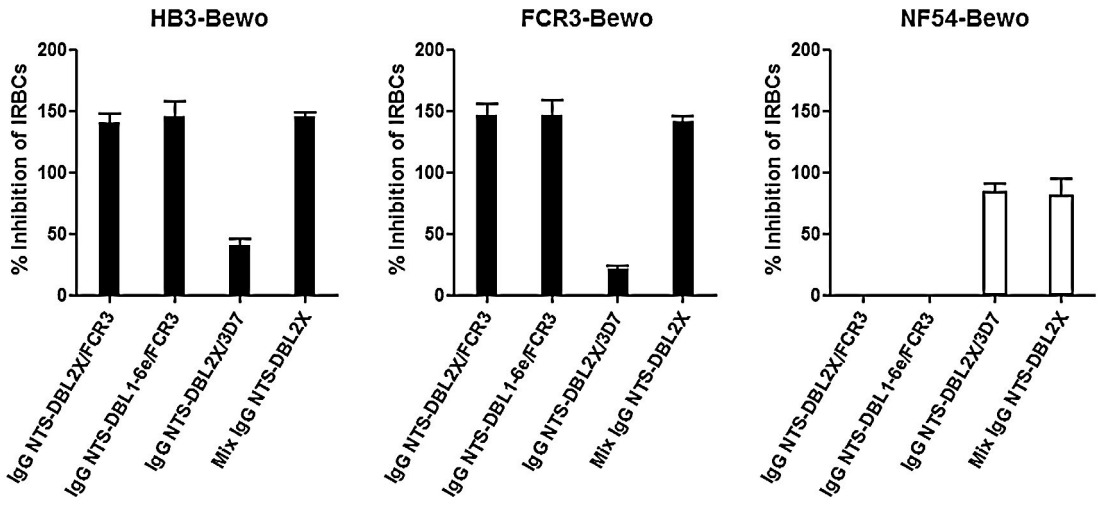

Fig. 4. Inhibition profile of antibodies on selected and laboratory-adapted CSA-binding strains. FCR3, HB3 and NF54 strains selected on Bewo-cells (FCR3-Bewo, HB3-Bewo and NF54-Bewo) were used to assess the ability of the induced antibodies to inhibit binding of 3 laboratory-adapted strains to CSPG. The *in vitro* functionality of the mixture of both antibodies was also evaluated. Values are normalized with CSA inhibition value.

more. In addition, the mixing of the two antibody preparations did not affect their individual activity. Analysis of isolates in which we observed strong contrasts in the properties of both types of antibodies clearly suggests that the DSM region is not the main target for adhesion inhibitory antibodies. A possible explanation might be that the sequences of these isolates significantly differ from their counterparts at other places. Thorough sequence and functional analysis of these isolates is underway.

In conclusion, this study has demonstrated that the NTS-DBL2X domain of both the FCR3 and 3D7 parasite strains is able to induce strain-transcendent adhesion-inhibitory antibodies. The combination of individual antibodies properties suggests that optimal inhibitory spectrum on field isolates might be achieved with antibodies targeting two variants of the N-terminal VAR2CSA. The results thus provide evidence on the relevance and the need for molecular optimization of the VAR2CSA-based vaccines currently in development. Conducting such studies on larger numbers of samples is needed to further refine these observations.

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Conflict of interests

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.07.072.

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Article VI: Functional antibodies against VAR2CSA in non-pregnant populations from Colombia exposed to *Plasmodium falciparum* and *Plasmodium vivax.*

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Les constructions tronquées de *var2csa* composées de fragments courts comme le ID1-DL2X représentent actuellement les constructions les plus prometteuses pour le développement d'un vaccin efficace contre le PG. Plusieurs études portant sur l'analyse des niveaux d'anticorps acquis naturellement contre VAR2CSA ont été réalisées en zone d'endémie (le plus souvent en Afrique). Ces travaux ont montré une distribution sexe et parité dépendance de ces anticorps. Les niveaux élevés de ces anticorps ont été également associés à la protection contre l'issue défavorable de la grossesse (77,78). En Amérique Latine, plusieurs pays dont la Colombie sont endémiques au paludisme avec une transmission faible et instable.

Dans cette étude, nous avons analysé chez les populations enceintes et non enceintes de la Colombie les niveaux d'anticorps spécifique de VAR2CSA et l'activité anti-adhérence de ces anticorps.

Les anticorps dirigés contre plusieurs constructions de VAR2CSA ont été détectés dans la population d'étude. Cependant des taux élevés ont également été observés chez des hommes et des enfants infectés par *P. falciparum* et *P. vivax*. Cette observation inattendue, notamment parmi les sujets non-enceints qui étaient uniquement exposés au *P. vivax,* diffère des données générées à partir de plusieurs autres pays endémiques, mettant en évidence le mode sexe et parité dépendante de l'acquisition des anticorps contre VAR2CSA. L'analyse des propriétés fonctionnelles de ces anticorps (capacité à inhiber l'adhérence des EI à la CSA) a permis de confirmer leur potentiel anti-adhérence sur des souches sélectionnées sur CSA. Ceci suggère une particularité génotypique et phénotypique des parasites de cette région géographique ou l'existence de facteurs génétiques de l'hôte, qui jouerait un rôle dans la modulation de la réponse immunitaire contre les infections plasmodiales.

Ce travail montre clairement une acquisition différentielle des anticorps dirigés contre VAR2CSA chez des sujets non-enceints en Amérique Latine, et ouvre de nouvelles perspectives sur la nécessité de caractériser cette immunité au-delà de la grossesse. L'expression de VAR2CSA ou l'existence d'épitopes similaires chez des parasites circulant dans les zones d'endémie palustre en dehors de l'Afrique, mérite d'autres investigations.

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Functional Antibodies against VAR2CSA in Nonpregnant Populations from Colombia Exposed to *Plasmodium falciparum* and *Plasmodium vivax*

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In pregnancy, parity-dependent immunity is observed in response to placental infection with *Plasmodium falciparum*. Antibodies recognize the surface antigen, VAR2CSA, expressed on infected red blood cells and inhibit cytoadherence to the placental tissue. In most settings of malaria endemicity, antibodies against VAR2CSA are predominantly observed in multigravid women and infrequently in men, children, and nulligravid women. However, in Colombia, we detected antibodies against multiple constructs of VAR2CSA among men and children with acute *P. falciparum* and *Plasmodium vivax* infection. The majority of men and children (>60%) had high levels of IgGs against three recombinant domains of VAR2CSA: DBL5ε, DBL3X, and ID1-ID2. Surprisingly, these antibodies were observed only in pregnant women, men, and children exposed either to *P. falciparum* or to *P. vivax*. Moreover, the anti-VAR2CSA antibodies are of high avidity and efficiently inhibit adherence of infected red blood cells to chondroitin sulfate A *in vitro*, suggesting that they are specific and functional. These unexpected results suggest that there may be genotypic or phenotypic differences in the parasites of this region or in the host response to either *P. falciparum* or *P. vivax* infection outside pregnancy. These findings may hold significant clinical relevance to the pathophysiology and outcome of malaria infections in this region.

Malaria is a major public health problem that disproportionately affects young children and pregnant women. Malaria during pregnancy is associated with placental infection, maternal anemia, low-birth-weight (LBW) infants, and increased neonatal morbidity and mortality (1). Pregnant women, especially primigravidae, are highly susceptible to malaria infection, despite preexisting immunity, as they are exposed to a specific subpopulation of *Plasmodium falciparum* (*P. falciparum*) that accumulates in the placenta (2). Women living in areas of intense or stable *P. falciparum* transmission are mostly asymptomatic but at risk of severe maternal anemia, placental infection, and negative birth outcomes such as stillbirth and fetal growth retardation. In these areas, the pathogenic effects of pregnancy-associated malaria (PAM) decrease with increasing parity as women acquire PAM-specific protective immunity (3). In areas of lower transmission, malaria infection is often symptomatic in women of all parities and associated with substantial malaria-related fetal loss and maternal death (4).

During pregnancy, specific variants of *P. falciparum*-infected erythrocytes (IEs) sequester in the placenta. This mechanism is mediated by an interaction between chondroitin sulfate A (CSA) on the syncytiotrophoblasts and variant surface antigens (VSA), expressed by the parasite on the surface of IEs (2). The VSA family includes *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (5), repetitive interspersed family (RIFIN) proteins (6), subtelomeric variable open reading frame (STEVOR) proteins (7), and surface-associated interspersed gene family (SURFIN) proteins (8).

VAR2CSA belongs to the PfEMP1 family and is the main parasite ligand that mediates placental binding (9). Knockout studies demonstrated the pivotal role of the *var2csa* gene in parasite adhesion to placental CSA (10). *var2csa* is selectively transcribed in both *P. falciparum* parasites isolated from the placenta (11) and parasite strains selected *in vitro* for adhesion to CSA (12). The *var2csa* gene is relatively conserved between PAM strains. VAR2CSA is a large protein (350 kDa) that is structurally composed of six Duffy binding-like (DBL) domains (DBL1X, DBL2X, DBL3X, DBL4ε, DBL5ε, and DBL6ε), a cysteine-rich region between DBL2X and DBL3X, and several interdomains (13, 14). Each DBL domain contains conserved and polymorphic regions that can be targeted by surface-reactive antibodies (15–17). Conserved regions are predominant in DBL3X, DBL4ε, and DBL5ε domains. Interestingly, pregnant women exposed to malaria mainly recognize the DBL3X and DBL5ε domains, suggesting that specific immune memory to these VAR2CSA domains is naturally acquired with exposure. Several single domains from VAR2CSA bind to CSA *in vitro*, including DBL3X, DBL5ε, and ID1-ID2, which spans the DBL2 domain (14, 16–19). However, a specific and high binding affinity to CSA depends on the folded architec-

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ture of the full VAR2CSA protein (20). VAR2CSA is predominantly recognized in a parity-dependent manner by antibodies present in plasma of pregnant women exposed to malaria. Importantly, high levels of antibodies against VAR2CSA are associated with protection from delivering LBW infants (21). Furthermore, naturally acquired antibodies to VAR2CSA can efficiently block IE adhesion to CSA (22, 23). These antibodies are occasionally detected in nonpregnant patients (24, 25); however, the prevalence and levels of anti-VAR2CSA antibodies are much lower than in pregnant women from the same region (24).

Most studies on PAM focus on women in sub-Saharan Africa where malaria transmission is high. However, malaria is also endemic in many countries in Latin America. In Colombia, malaria transmission is low and unstable throughout the country. Over 100,000 cases are reported every year (26), and both *P. falciparum* and *Plasmodium vivax* (*P. vivax*) are prevalent (27). Intermittent preventive treatment in pregnancy (IPTp) using sulfadoxine/pyrimethamine has not been implemented in Colombia, and attendance at antenatal clinics is low. In a recent study, we demonstrated a high rate of submicroscopic malaria infections in asymptomatic pregnant women at delivery using sensitive molecular diagnostics. Despite a high frequency of infection (60%), no negative birth outcomes were observed (28).

Here, we investigated the levels, specificity, and antiadhesion activity of VAR2CSA antibodies among pregnant and nonpregnant populations from Colombia to determine exposure to placental parasite antigens in this region.

MATERIALS AND METHODS

Ethical approval. The study was approved by the Health Research Ethics Board of the University of Alberta (Canada), the Comité de Ética of Instituto de Investigaciones Médicas (Universidad de Antioquia, Colombia), the Comité Consultatif de Déontologie et d'Ethique of the Research Institute for Development (France), and the ethical committee of the Faculty of Health Science (University of Abomey-Calavi, Benin). All procedures complied with Canadian, European, Colombian, and Beninese national regulations.

Study region. Study participants exposed to malaria (pregnant women, children, and men) were recruited at the local obstetric facility or malaria clinic of the municipality of Puerto Libertador (07°54′N, 75°40′W) in the Department of Córdoba, northwest Colombia. The Department of Córdoba is within the malaria transmission region termed Urabá-Altos Sinú-San Jorge-Bajo Cauca. This region accounts for 60% of all malaria cases in Colombia (26). The entomological inoculation rate in this region ranges from 3.5 to 4.8 infective bites per person per year (29). *P. vivax* is reported in approximately 70% of cases, based on diagnosis by microscopy. The mean annual parasitic index (number of malaria cases/1,000 inhabitants) during 2000 to 2009 in Puerto Libertador was 23.4 (29).

Study design and sample collection. Pregnant women with acute malaria (quantitative PCR [qPCR] positive) and without malaria (qPCR negative) were enrolled either during their second or third trimester of pregnancy or at delivery. Children (≤15 years old) and men who presented to the malaria clinic in Puerto Libertador with acute, uncomplicated malaria were recruited. Twenty-five women and 25 men without a history of malaria or travel to an area of malaria endemicity (nonexposed) were recruited in the city of Medellín (6°13′55″N, 75°34′05″W), a municipality free of malaria transmission.

Blood samples (4 to 5 ml) were collected by venipuncture from each volunteer. Thick and thin blood smears were prepared for microscopy, and blood was spotted onto filter paper for DNA extraction. Malaria was diagnosed as described elsewhere (28). In brief, field-stained thick smears were read by an experienced microscopist in the local laboratory. Microscopy analysis was followed by genomic DNA extraction from the filter spots using the saponin-Chelex method (30). Real-time quantitative PCR (qPCR) was performed as described elsewhere (31). Serum was separated from the collected blood by centrifugation and stored at −20°C until processed.

Sera from Beninese women collected in a previous study (32) served as positive controls for VAR2CSA antibody levels and antiadhesion activity. Briefly, malaria transmission in Benin is high and peaks during two rainy seasons. *P. falciparum* is the predominant species, and the entomological inoculation rate ranges from 35 to 60 infective bites per person per year (33). Sera from 30 primigravid women and 30 multigravid women that had been previously characterized for their reactivity against VAR2CSA protein were used in this study. Controls included serum samples from 25 Beninese men and children exposed to malaria that were collected in a previous study (33) and sera from 20 Canadian adults without malaria exposure.

Antibody analysis assay. The ID1-ID2, DBL3X, and DBL5ε domains of VAR2CSA from *P. falciparum* strain FCR3 were produced in baculovirus-infected SF9 cells, as described previously (14, 16) (34). Optimal concentrations (0.5 μg/ml) of each protein were coated onto Maxisorb microtiter plates. The specific levels of IgG were measured in serum samples using an enzyme-linked immunosorbent assay (ELISA) as described elsewhere (35). Briefly, the recombinant proteins were incubated with 100 μl of human sera at a dilution of 1:1,000 followed by horseradish peroxidase-conjugated anti-human IgG (1:6,000) to measure total IgG. Twenty sera from Canadian residents with no history of travel to areas of malaria endemicity served as negative controls. A pool of serum samples from multigravid women from Benin, previously demonstrated to have high levels of anti-VAR2CSA IgG against placental isolates, served as a positive control. Optical density (OD) values were converted into arbitrary units, as described previously (35). Antibody responders were defined as those having an antibody level (in arbitrary units [AU]) of >2 standard deviations (SD) above the mean absorbance of the negative controls.

All sera were tested for prior exposure to *P. falciparum* and *P. vivax* using recombinant *P. falciparum* merozoite surface protein 1 (PfMSP1; CTK-Biotech), *P. falciparum* glutamate-rich protein (PfGLURP) (36), *P. vivax* merozoite surface protein 1 (PvMSP1; CTK-Biotech), and *P. vivax* apical membrane antigen 1 (PvAMA1). ELISAs were used as described above except that sera were diluted at 1:8,000 for PfMSP1, 1:6,000 PvMSP1, and 1:1,000 for PvAMA1 and PfGLURP. A patient was regarded as exposed to a specific *Plasmodium* species if the serum reacted positively against at least one of the species-specific antigens. An ELISA endpoint titration assay was performed as described above, except that the DBL5ε recombinant protein was incubated with 100 μl of different pools of human sera at different dilutions (1:100, 1:200, 1:400, 1:500, 1:600, 1:1,000, 1:2,000, 1:4,000, 1:5,000, 1:10,000, 1:20,000, 1:40,000, 1:50,000, and 1:100,000). The antibody titer for each pool was determined based on the highest dilution at which the OD is 2 SD above the mean from the Canadian control at a dilution of 1/100 (0.793).

Competitive ELISA. Rabbits were genetically vaccinated with *var2csa* DNA. Antiserum against the full VAR2CSA protein was collected 75 days after the first immunization (day 75) as described in a previous study (23). Microtiter plates were coated with recombinant DBL5ε at a concentration of 0.5 μg/ml. Plates were blocked with 1× phosphate-buffered saline (PBS), 0.5 M NaCl, 1% Triton X-100, and 1% bovine serum albumin (BSA) for 4 h at room temperature (RT). Increasing dilutions (1:100, 1:1,000, 1:10,000, and 1:100,000) of the competing sera were added and incubated overnight at 4°C. The pool of sera from nonexposed Colombians served as the negative control. After samples were washed four times with PBS-Tween 20 at 0.1%, a fixed dilution (1:400) of noncompeting serum was added and incubated for 1 h at RT. A specific secondary antibody conjugated to horseradish peroxidase (either a goat anti-human [A0170, Sigma-Aldrich] or goat anti-rabbit [656120, Sigma-Aldrich]) directed against the noncompeting antibody diluted at 1:6,000 was added and incubated for 1 h at RT. After four washes, antibody reactivity of the

TABLE 1 General characteristics of the study population in Colombia

Category and pathogen	Sample size (no.)	Age (yr)[a]	No. with acute infection[b]	Parasite count/μl (range)[c]
Pregnant women	94[d]	21 ± 6 (13–38)		
P. vivax			38	3,513 (77–28,028)
P. falciparum			14	1,383 (160–30,109)
Men	57	33 ± 12 (19–70)		
P. vivax			28	4,732 (604–29,126)
P. falciparum			29	2,744 (554–82,880)
Boys	37	11 ± 2 (6–14)		
P. vivax			26	4,092 (118–14,700)
P. falciparum			11	2,616 (836–10,827)
Girls	20	9 ± 2 (5–14)		
P. vivax			10	3,608 (881–15,800)
P. falciparum			10	4,347 (769–19,670)

[a] Mean ± SD (range).
[b] Based on qPCR diagnosis.
[c] The geometric mean parasite density of patients with microscopic infections.
[d] Of these, 42 were not infected.

TABLE 2 Characteristics of the pregnant population

Category (*n*)	Infection profile (no. of women)			
	Not infected	Acute infection[a]	*P. vivax* infection	*P. falciparum* infection
Primigravid (15)	12	3	2	1
Multigravid (40)	28	12	6	6
Other (39)[b]	2	37	30	7

[a] Based on qPCR diagnosis.
[b] Gravidity not known.

noncompeting plasma/serum was visualized at 450 nm following the addition of tetramethylbenzidine (TMB; Sigma-Aldrich). The percent reduction in antibody reactivity in the presence of a competitor was calculated as follows: 100 × (OD with competitor antibody/OD without competitor antibody) (23).

Avidity assays. The avidity of anti-DBL5ε antibodies was assessed with a urea elution-based ELISA. Microtiter plates were coated with recombinant protein as described above. Sera (1:1,000) were incubated in quadruplicate wells and incubated overnight at 4°C and then washed three times with PBS-Tween 20 at 0.1%. Duplicate wells were incubated for 15 min with either urea (8 M) or 1× PBS. The plates were washed with PBS-Tween 20 at 0.1%. Incubation with the secondary antibody and developing enzyme reactions were performed as described above for ELISAs. The avidity index (AI) was calculated as the ratio of the OD value of urea-treated samples to that of the untreated samples, multiplied by 100. All AI values less than 30% were considered low-avidity antibodies, values between 30% and 50% were considered intermediate-avidity antibodies, and values greater than 50% were considered high-avidity antibodies (37).

IgG preparation. Total IgG was purified from human sera on a HiTrap protein G high pressure (HP) column according to the manufacturer's recommendations (GE Healthcare). In brief, 500 μl of sera was diluted in 20 mM sodium phosphate buffer (pH 7.4), applied to a preequilibrated column (17-0404-03; GE Healthcare), and incubated for 1 h at room temperature. After coupling, unbound proteins were washed through the column with 10 volumes of 20 mM sodium phosphate buffer (pH 7.4). Bound IgG was eluted with 0.1 M glycine-HCl (pH 2.7) and neutralized with 1 M Tris-HCl (pH 9). Eluted fractions were dialyzed against 1× PBS and concentrated using Amicon centrifugal filter devices (10,000 kDa; Millipore). Purified IgG was used for Western blot analysis and parasite adherence inhibition assays.

Inhibition of IE binding to CSPG by specific IgG. The static assay employed to evaluate the capacity of the antibodies to interfere with CSA-specific adhesion of IEs is described in detail elsewhere (38). *P. falciparum* parasite strains FCR3 and HB3 were repeatedly panned on the human choriocarcinoma cell line BeWo (FCR3-BeWo and HB3-BeWo, respectively), as described previously (39). In this assay, a petri dish (351029; Becton, Dickinson) was coated overnight at 4°C with 20 μl of ligand, 1% BSA, and 5 μg/ml chondroitin sulfate proteoglycan (CSPG) (decorine; Sigma) diluted in PBS. Each spot was subsequently blocked with 3% BSA in PBS for 30 min at RT. Enriched late-stage-infected IEs were also blocked in 3% BSA-RPMI medium for 30 min at RT. Parasite suspensions adjusted to 20% parasite density were incubated with serum (1:5 dilution), purified IgG (250 μg/ml final concentration), or 500 μg/ml soluble CSA for 30 min at RT before they were added to the ligand and incubated for 15 min at RT for binding. Nonadherent cells were removed by an automated washing system. Spots were fixed with 1.5% glutaraldehyde in PBS and stained with Giemsa. Adherent IEs were quantified by microscopy as the number of IEs bound per millimeter squared, estimated from 20 high-power fields (40).

Western blot assay. Recombinant VAR2CSA DBL5ε protein (2 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred onto 0.2-mm-pore-size Protan BA 83 nitrocellulose sheets (Invitrogen) for immunodetection. The membrane was blocked for 1 h with 5% nonfat dry milk in 1× PBS with Tween 20 at 0.1% and then incubated separately with a 1:2,000 dilution of purified IgG from Beninese multigravid women and a 1:1,000 dilution of purified IgG from Canadian controls or unexposed Colombian controls or from Colombian men, children, or pregnant women. After three washes, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody (1:15,000 dilution) (A0170; Sigma-Aldrich). Bound antibody was detected with enhanced chemiluminescence (ECL) substrate (GE Healthcare Life Sciences).

Statistical analyses. Each sample was tested in duplicate and run in two independent experiments. Comparison of anti-VAR2CSA antibody levels between groups was performed using nonparametric Mann-Whitney/Wilcoxon and Kruskal-Wallis tests. A chi-square test was used to examine differences between categorical variables. Comparison of serological recognition of specific antigens by sera from different groups of patients was done similarly. Data were plotted using Prism software (version 6; GraphPad). EPI Info software (version 3.5.3) and Prism software (version 6; GraphPad) were used for statistical analyses. P values of <0.05 were considered to be statistically significant.

RESULTS

Characteristics of participants. The general characteristics of the different study populations are shown in Table 1. Fifty-seven men and 57 children from Colombia, all with acute malaria infection, were tested in the present study. Ninety-four pregnant women from Colombia were included in this study: 42 were not infected, 38 were positive for *P. vivax*, and 14 were positive for *P. falciparum* (Table 1). Among the 94 women, 15 were primigravid, and 40 were multigravid. The parity of the other 39 women was unknown. The detailed characteristics of the pregnant women are presented in Table 2.

Children, men, and pregnant women from Colombia have antibodies that recognize multiple domains of *P. falciparum* VAR2CSA. Based on studies primarily in Africa, it is well established that antibody levels against VAR2CSA increase with parity and correlate with acquired immunity to placental parasites. We first compared the levels of antibodies to the DBL5ε domain of VAR2CSA in pregnant women of different parities from Benin and Colombia (Fig. 1A). As expected, Beninese multigravid women had higher antibody levels against DBL5ε than primigravid women ($P < 0.0001$). In Colombia, the mean antibody

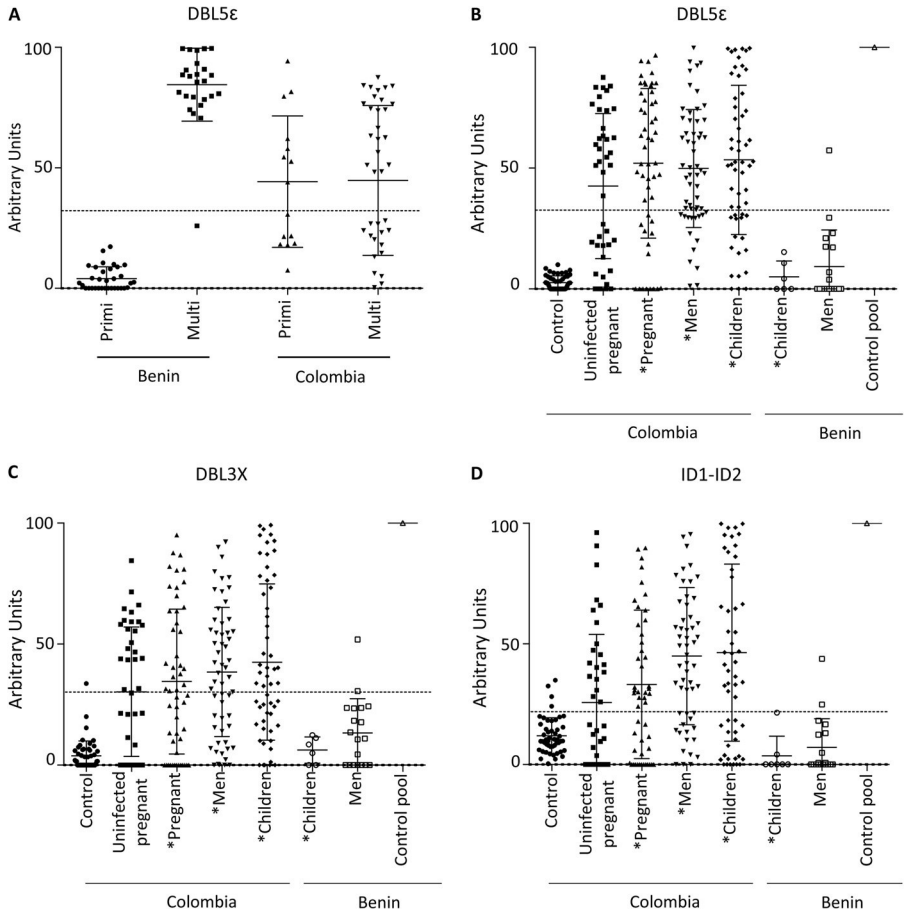

FIG 1 Sera from Colombian pregnant women, men, and children recognize several domains of VAR2CSA. DBL5ε antibody levels were quantified in primigravid (Primi) and multigravid (Multi) women from Benin and Colombia (A). Sera with specificity for DBL5ε (B), DBL3X (C), and ID1-ID2 (D) domains of VAR2CSA were measured in unexposed Colombians (Control), pregnant women with and without acute infection, men and children from Colombia, and men and children from Benin. A pool of sera from multigravid women from Benin (Control pool) served as a positive control. Sera were diluted 1:1,000. Data are expressed as arbitrary units. Bars indicate the means ± standard deviations. The experiment was performed twice with similar results. The cutoff level for classification as positive is represented by the horizontal stippled line. *, patients with acute malaria infection.

level was lower than in pregnant women from Benin, and there was no difference between primigravid and multigravid women ($P = 0.2272$). Moreover, the levels of anti-DBL5ε antibodies in Colombian pregnant women with or without a malaria infection were similar ($P = 0.1278$) (Fig. 1B).

Unexpectedly, men and children also had antibodies against the DBL5ε domain at similar levels ($P = 0.3455$) and frequencies (66%) to pregnant women (Fig. 1B). This is contrary to reports that antibodies to VAR2CSA are rarely observed in nonpregnant populations. Furthermore, men and children had antibodies that recognized two other domains of VAR2CSA, DBL3X and ID1-ID2, also with high frequencies (men, 58% DBL3X and 70% ID1-ID2; children, 56% DBL3X and 65% ID1-ID2) (Fig. 1C and D). The VAR2CSA antibody levels detected in the Colombian nonpregnant populations were higher than those quantified in Beninese nonpregnant populations (DBL5ε, $P < 0.0001$; DBL3X, $P < 0.0001$; ID1-ID2, $P < 0.0001$).

This antigen recognition is specific to malaria exposure since adults residing in the malaria-free city of Medellín did not have antibodies against these antigens, nor did the Canadian control group. These results were further confirmed by Western blot analysis that showed that total IgG purified from a pool of sera from either Colombian children or men recognized the purified, recombinant DBL5ε (Fig. 2A). The same product (37 kDa) was recognized by specific IgGs purified from Colombian pregnant women, as well as Beninese multigravid women. No band of the expected size was observed with purified IgG from either unexposed Canadians or Colombians.

Despite the pivotal involvement of VAR2CSA in PAM, men and children had antibodies against the VAR2CSA domains with comparable levels to those observed in pregnant women. All three populations had similar levels of anti-DBL5ε ($P = 0.6206$) and anti-DBL3X antibodies ($P = 0.1218$) (Fig. 1B and C). Interestingly, men and children had higher ID1-ID2 antibody levels than pregnant women ($P = 0.0007$) (Fig. 1D). We further compared VAR2CSA antibody levels in Colombian patients with those in Beninese multigravid women. Sera from all samples with positive reactivity against DBL5ε were pooled and serially diluted (Fig. 2B). A pool of sera from Canadian controls was used to define the endpoint titer for each patient group. Endpoint titers were similar for Colombian pregnant women and men (dilution of 1/1,000) while the endpoint titer of the sera from Colombian children was

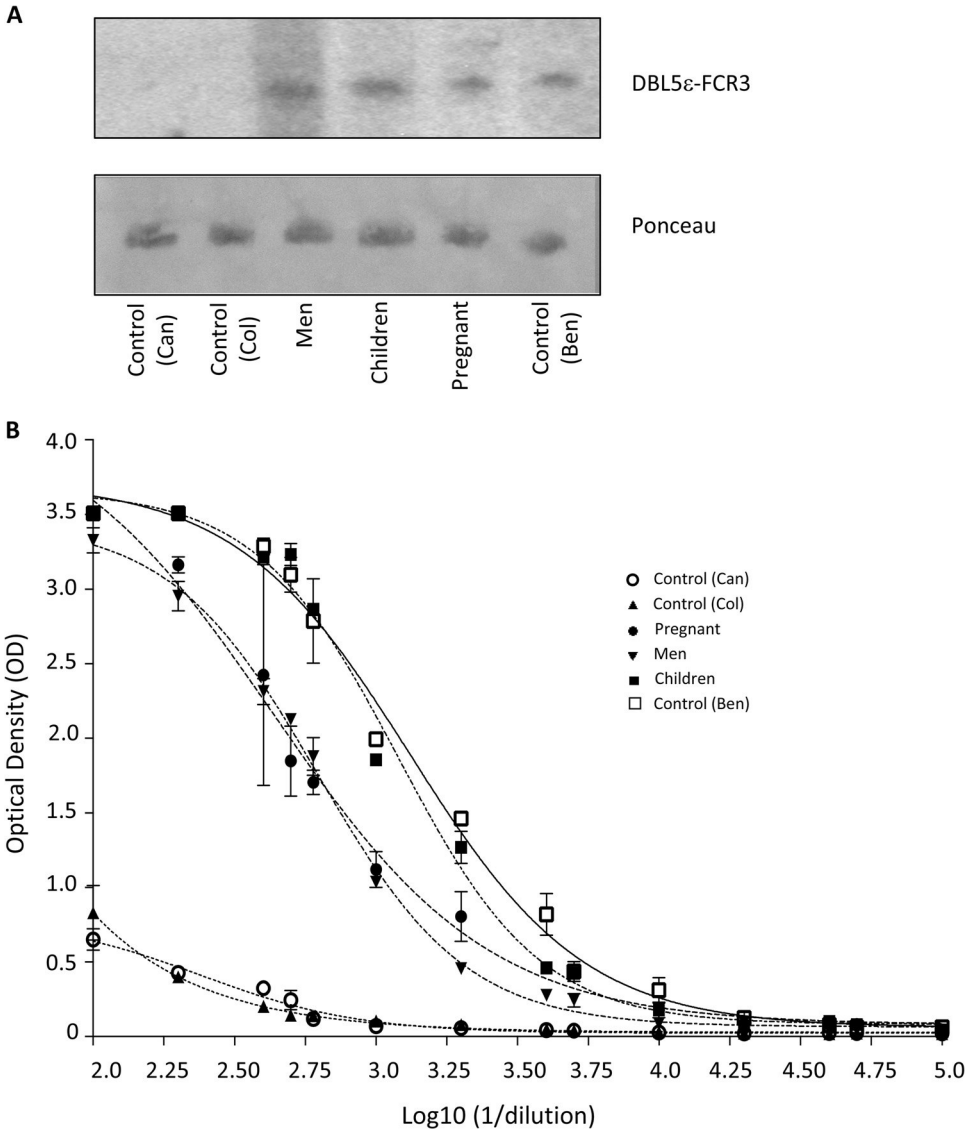

FIG 2 VAR2CSA antibody levels in Colombian populations. (A) SDS-PAGE with DBL5ε (2 μg) immunoblotted with purified IgG from pregnant women, men, and children from Colombia. IgG from Beninese multigravid women (Control Ben) served as a positive control, and unexposed adults from either Canada (Can) or Colombia (Col) served as negative controls. (B) Sera from Colombian pregnant women, men, and children with DBL5ε reactivity by ELISA were pooled and serially diluted. Endpoint titers were determined based on a 1/100 dilution of sera from unexposed Canadians. Assays were performed in duplicate, and values are means ± standard deviations. Experiments were performed twice with similar results.

lower (1/2,000). The titer from the Beninese multigravid women was 1/4,000.

Anti-VAR2CSA antibodies in patients with acute or prior exposure to *P. falciparum* and *P. vivax*. All of the men and children in our study had an acute malaria infection. A possible explanation for the high anti-VAR2CSA antibodies is that *P. falciparum* strains in Colombia indiscriminately express *var2csa* outside pregnancy. We therefore asked whether reactivity to the VAR2CSA domains correlated specifically with *P. falciparum* acute infection. While 76% of children, 79% of men, and 57% of pregnant women infected with *P. falciparum* had antibodies against VAR2CSA, a similar proportion had an acute infection with *P. vivax* (Table 3). Comparable results for the two species were observed for all three VAR2CSA domains ($P > 0.05$).

Alternatively, these antibodies may have been acquired during a prior exposure to *P. falciparum*. Exposure to *P. falciparum* or *P. vivax* was determined based on the levels of antibody to several surface antigens that are specific for each species (PfMSP1, PfGLURP, PvMSP1, and PvAMA1). More than 50% of pregnant women and 70% of men and children exposed to only *P. falciparum* reacted against DBL5ε (Table 4). Unexpectedly, the same reactivity was observed in patients with sera reactive only to antigens from *P. vivax*.

High-avidity antibodies against VAR2CSA among sera from Colombian men, children, and pregnant women. Although men and children exhibited high levels of antibodies to DBL5ε, the avidity of the antibodies for the antigen is an important indication of their specificity. The avidity of IgG antibody to DBL5ε was examined in positive sera ($n = 61$ for pregnant women, $n = 38$ for men, and $n = 40$ for children) using 8 M urea as a dissociation

TABLE 3 Relationship between Colombian samples that positively reacted against domains of VAR2CSA and acute infection

Category and pathogen (*n*)[a]	No. with acute infection	% of samples reactive to the indicated VAR2CSA domain		
		DBL5ε	DBL3X	ID1-ID2
Pregnant women (94)				
P. vivax	38	76	55	57
P. falciparum	14	57	36	50
Not infected	42	57	50	43
Men (57)				
P. vivax	28	53	50	61
P. falciparum	29	79	65	79
Children (57)				
P. vivax	36	67	58	64
P. falciparum	21	76	52	66

[a] *n*, number in group.

TABLE 5 Frequency of high-, intermediate-, and low-avidity antibodies to DBL5ε in Colombian patients with acute malaria infection

Patient group (*n*)	Antibody avidity (% of samples)[a]		
	HAI	IAI	LAI
Pregnant women (61)	80	20	0
Men (38)	74	13	13
Children (40)	75	17.5	7.5

[a] High avidity index (HAI), >50% of control; intermediate avidity index (IAI), 30 to 50% of control; low avidity index (LAI), <30% of control.

agent. High-avidity IgG was observed in 80% of pregnant women, 74% of men, and 75% of children. Twenty percent of pregnant women had intermediate-avidity antibodies compared to 13% of men and 18% of children (Table 5). Antibodies with low avidity were detected in 13% and 8% of men and children, respectively, but not in pregnant women. No significant correlation was observed between antibody avidity and acute infection (data not shown).

Antibodies from pregnant women, men, and children from Colombia as well as from Beninese multigravid women share common VAR2CSA epitopes. The ELISA and Western blot assays demonstrated that nonpregnant and pregnant subjects from Colombia had antibodies against recombinant domains of VAR2CSA. We further tested the specificity of these antibodies for epitopes within the full-length VAR2CSA protein using a competitive ELISA approach. The DBL5ε domain of VAR2CSA was used in a competitive ELISA to analyze the target epitopes of naturally acquired antibodies from patient sera and rabbit antiserum against the full VAR2CSA protein. Men and children as well as pregnant women from Colombia presented the same inhibition patterns in binding to the recombinant DBL5ε protein in the presence of increasing concentrations of the rabbit antiserum (Fig. 3A). Similar inhibition was observed when rabbit anti-VAR2CSA antiserum competed with antibodies from exposed Colombian subjects and multigravid women from Benin (Fig. 3B). No competition for binding was observed with the sera from unexposed Colombians.

IgG from Colombian populations inhibits adhesion of infected *P. falciparum* erythrocytes to CSA. One of the hallmarks of VAR2CSA antibodies in pregnancy is their ability to inhibit parasite adhesion to CSA. Using a static *in vitro* assay, we tested whether antibodies from the Colombian populations can inhibit parasite adhesion to CSA. As observed in studies of pregnant women from Africa, most sera from Colombian pregnant women (80%) inhibited adhesion to *P. falciparum* laboratory strains FCR3 repeatedly panned on BeWo cells (Fig. 4A). Surprisingly, a high proportion of sera from men (65%) and children (90%) also blocked adhesion to FCR3-BeWo (Fig. 4A). No inhibition was observed with the pool of sera from Canadians. As expected, the pool of sera from Beninese multigravid women demonstrated a strong inhibitory effect on both parasites strains.

Several studies demonstrated that nonspecific IgM antibodies can inhibit parasite adhesion (41, 42). To exclude this possibility, total IgG was purified from pools of sera from 6 men, 9 children, and 10 pregnant women. Samples with antiadhesion activity on CSA-binding parasites (Fig. 4A) were selected for each pool. Purified antibodies from all three groups demonstrated antiadhesion activity. This activity was observed on both FCR3-BeWo and HB3-BeWo parasite lines (Fig. 4B). This result confirms that the inhibition mechanism is mediated by naturally acquired IgG.

DISCUSSION

One of the key findings in this study is that pregnant women, men, and children from Colombia have high frequencies and levels of antibodies against *P. falciparum* VAR2CSA. Moreover, these antibodies can inhibit parasite adhesion to CSA *in vitro*. It was reported in previous studies that antibodies against VAR2CSA are not exclusively restricted to pregnancy (24, 25). However, the level and prevalence of the antibodies observed in those studies were lower in men and children than in exposed multigravid women (24). From proteomic studies, VAR2CSA was detected in parasites of nonplacental origin (43), and in another study, parasites from nonpregnant hosts could bind to CSA (24, 44). Consistent with our study, widespread DBL5ε reactivity was observed in plasma from Tanzanian children, suggesting that VAR2CSA in nonpregnant populations could share specific epitopes with VAR2CSA from placental parasites (25). The high proportion of anti-VAR2CSA antibodies detected in our Colombian populations in-

TABLE 4 Antibody reactivity to DBL5ε and malaria exposure in Colombian pregnant and nonpregnant populations

Pathogen exposure (surface antigen)	Pregnant women (*n* = 94)		Men (*n* = 57)		Children (*n* = 57)	
	No. infected	% DBL5ε^{+}[a]	No. infected	% DBL5ε^{+}	No. infected	% DBL5ε^{+}
P. vivax (PvMSP1 or PvAMA1)	59	69	25	56	30	70
P. falciparum (PfMSP1 or PfGLURP)	2	50	7	71	10	70
Both species	18	78	23	78	12	75
Not exposed	15	33	2	50	5	60

[a] Percentage indicates the proportion of samples that reacted against DBL5ε (DBL5ε^{+}) related to exposure to *P. vivax* and *P. falciparum*.

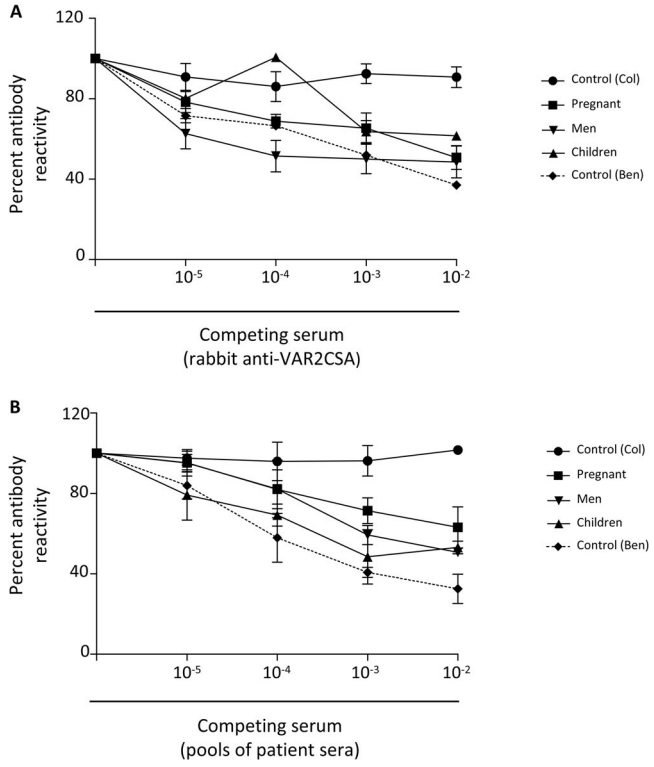

FIG 3 Competitive recognition of recombinant DBL5ε-VAR2CSA between rabbit antibodies against VAR2CSA and naturally acquired antibodies from Colombian and Beninese subjects. (A) Pools of sera (1:400) from Colombian unexposed controls, pregnant women, children, and men and from Beninese multigravid women were competed with increasing concentrations of day 75 VAR2CSA antiserum from a vaccinated rabbit (competing serum). (B) Day 75 VAR2CSA antiserum from a vaccinated rabbit (1:80,000) was competed with increasing concentrations of pooled sera from Colombian unexposed controls, pregnant women, children, and men and from Beninese multigravid women (competing sera). The error bars show the standard deviations of two independent wells. Experiments were performed twice with the same results. Antibody reactivity is expressed relative to the OD in the absence of competing serum as described in Materials and Methods.

dicates that low malaria transmission in this area is sufficient to induce high levels of VAR2CSA antibodies outside and during pregnancy.

The high frequency of VAR2CSA antibodies in Colombian populations suggests that exposure to VAR2CSA or to parasites expressing VAR2CSA-like antigens may be common in the general population. A plausible hypothesis is that exposure to these VAR2CSA-like antigens in Colombia may arise from the cocirculation of *P. falciparum* and *P. vivax* (26). In our study region, patients exposed to either *P. falciparum*, *P. vivax*, or both species had VAR2CSA antibodies. Other DBL-containing proteins from *P. falciparum* (other PfEMP1s) or from *P. vivax* may generate cross-reactive antibodies. Ours is the first study to suggest that epitopes from *P. vivax* antigens may cross-react with VAR2CSA from *P. falciparum*. Study of VAR2CSA expression and function in other countries where *P. vivax* or both *Plasmodium* species circulate will further characterize the *var2csa* gene and protein function in regions outside Africa.

It is also possible that the *var2csa*-expressing strains in Colombia are genetically or phenotypically different from parasites encountered in Africa. A study of the evolution of *P. falciparum* strains from their African origins to their introduction in South America revealed highly differentiated parasite populations in northwestern Colombia (45). These parasites may be characterized by sequence variation within the *var2csa* gene or have altered mechanisms of VAR2CSA protein expression, regulation, and immunogenicity resulting in expression of *var2csa* outside pregnancy. DNA sequence and expression analysis of *var2csa* in parasites isolated from this region may reveal important differences in genetic control at this locus compared with African isolates. Other contributing factors may relate to the geography of our study region. Many infectious diseases are prevalent in this population, and other pathogens could potentially express antigens that induce cross-reactive antibodies to VAR2CSA. Consistent with this hypothesis, we identified a few patients exposed neither to *P. falciparum* nor *P. vivax* who nevertheless had antibodies to DBL5ε. Alternatively, the genetics of the host population, which is largely of indigenous descent, could also play a role in modulating the immune response to malaria infection.

Our data suggest that populations from Colombia are exposed

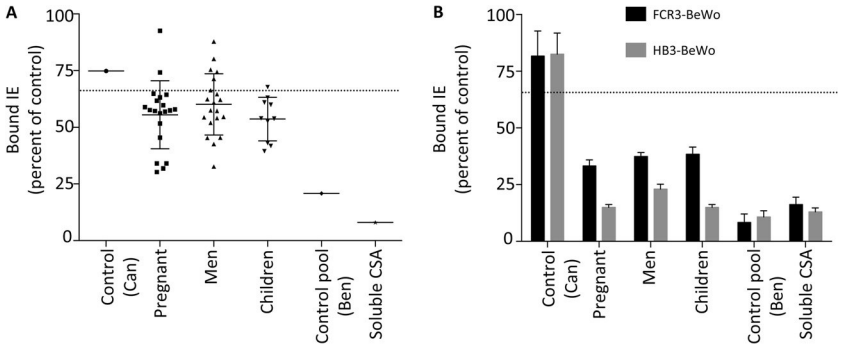

FIG 4 Inhibition of adhesion of *Plasmodium falciparum*-infected erythrocytes to CSA by sera from pregnant women, men, and children from Colombia. (A) Individual serum samples from pregnant women ($n = 20$), men ($n = 20$), and children ($n = 10$) from Colombia with positive reactivity to DBL5ε inhibited binding to CSA of FCR3-BeWo-infected erythrocytes (IE) expressing VAR2CSA. A pool of Canadian nonimmune sera served as a negative control (Control Can). A pool of sera from Beninese multigravid women served as a positive control (Control Ben). Soluble CSA was used as the competitor. (B) Purified IgGs from Colombian pregnant women, children, and men specifically inhibited binding of FCR3-BeWo and HB3-BeWo IEs to CSA. Purified IgGs from unexposed Canadians (Control Can), Beninese multigravid women (Control Ben), and soluble CSA served as controls. Each point represents the mean of duplicate wells ± standard deviations. Each experiment was performed twice with similar results.

to parasite variants, possibly expressing particular *P. falciparum* proteins, such as PfEMP1 alleles or *P. vivax* proteins that share similar or cross-reactive epitopes with VAR2CSA. An important consideration is whether these antibodies are protective. The current thinking is that antibodies against VAR2CSA are acquired in a parity-dependent manner, and high levels of antibodies are associated with improved birth outcomes (46). We were unable to observe parity-dependent effects on anti-VAR2CSA IgG levels in our pregnant population given the small sample size of pregnant women with known parity. Instead, we observed that pregnant women, men, and children all had high levels of antibodies. These data suggest that high levels of antibodies against VAR2CSA do not lead to subsequent protection against PAM. Moreover, we did not observe an association between the levels of VAR2CSA antibodies and parasite densities (data not shown). Infections in Colombia are generally characterized by low parasitemia, but whether VAR2CSA antibodies contribute to this remains to be explored. Even if these antibodies are not fully protective, they may curb parasite density and limit anemia, which may explain the benign clinical outcomes of malaria observed previously in this region (28). This hypothesis is consistent with a recent study in Papua New Guinea in which high levels of malaria-specific IgG did not prevent infection but controlled acute parasitemia (47).

Nearly all of the studies on VAR2CSA have focused on sub-Saharan Africa, which bears the largest burden of pregnancy-associated malaria (11, 17, 21, 23). This study provides unique insight into acquired antibodies to VAR2CSA in pregnant and nonpregnant populations in a Latin American setting. These data prompt further studies on *var2csa* gene expression and function in areas of malaria endemicity outside Africa and suggest additional roles for this antigen in malaria immunity beyond pregnancy.

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Article en cours de publication :

Analyse du polymorphisme des séquences du segment N-terminale de VAR2CSA exprimées par les isolats de terrain.

Introduction

Le paludisme placentaire est la conséquence majeure de la séquestration des érythrocytes infectés par *Plasmodium falciparum* dans le placenta. Il est clairement établi que la protéine VAR2CSA joue un important rôle dans le mécanisme moléculaire de cette séquestration qui est la manifestation de l'interaction entre les protéines parasitaires exprimées à la surface des EI et les récepteurs placentaires dont le plus important est la CSA. Les résultats de plusieurs travaux visant à caractériser le gène codant pour cette protéine membre de la famille des protéines PfEMP1 ont conforté la possibilité du développement d'un vaccin contre le paludisme placentaire à base de VAR2CSA. Il ressort également de ces études que la région N-terminale de cette protéine composée de plusieurs domaines 1°) contient le site minimal de fixation à la CSA et 2°) a une forte capacité d'induction d'anticorps anti-adhérence. Ces observations suggèrent que les constructions vaccinales à partir de cette région attractive de VAR2CSA pourraient avoir une activité protectrice optimale. Toutefois, le niveau élevé de polymorphisme de séquences de VAR2CSA observé sur les isolats de terrain constitue un obstacle majeur dans la mise au point d'un vaccin à large spectre d'activité sur les souches de terrain. Récemment, il a été démontré qu'une plus grande proportion d'isolats de parasites serait ciblé par une approche de vaccin multivalent comprenant les génotypes FCR3 et 3D7 ou plus (185). L'hypothèse développée dans ce travail propose qu'un cocktail antigénique comportant, en plus des deux précédents variants identifiés, d'autres variants majeurs de VAR2CSA détectés à partir des isolats de terrain augmenterait l'efficacité vaccinal sur les isolats placentaires. Dans cette étude, nous avons analysé le polymorphisme de séquence dans la partie N-terminale de VAR2CSA exprimée par les isolats de *P. falciparum* qui infectent les femmes au cours de la grossesse et étudié la relation entre un génotype particulier et la capacité du parasite à se fixer à la CSA, la densité parasitaire, des facteurs liés à la mère et l'issue de la grossesse.

Matériel et méthode

Site d'étude et collecte des isolats de P.falciparum

La collecte des échantillons a été réalisée au cours d'études conduites entre 2008 et 2013 dans plusieurs centres de santé situés dans le sud du Bénin où la transmission du paludisme à *P. falciparum* est hyper-endémique (185–187). Un Test de Diagnostic Rapide (TDR) a été réalisé sur des femmes enceintes au cours de consultations prénatales et à l'accouchement. En

cas de test positif, les isolats de *P. falciparum* ont été collectés après la signature d'un consentement éclairé. Un frottis et une goutte épaisse ont été réalisés sur ces échantillons pour confirmer l'infection à *P. falciparum* et déterminer la densité parasitaire. Ces travaux ont été réalisés selon un protocole validé par le comité d'éthique de la Faculté des Sciences de la Santé de l'Université d'Abomey-Calavi (Bénin).

Les prélèvements sanguins collectés auprès des femmes parasités par *P. falciparum* ont été centrifugés et une partie du culot globulaire a été lavé et placé directement dans 9 volumes de Trizol puis conservé à -80°C pour une future extraction de l'ARN. Une autre partie du culot globulaire a été mis en culture pour la maturation des formes « ring » des parasites en trophozoïtes âgés ou schizontes jeunes. Le reste du culot globulaire a été conservé à -20°C pour l'extraction de l'ADN. Après coloration au Giemsa, les frottis et gouttes épaisses ont été lus et la parasitémie a été réalisée selon les recommandations de l'OMS. Les formes asexuées de *P. falciparum* et les leucocytes ont été comptés jusqu'à atteindre 500 parasites ou leucocytes. La densité parasitaire a été estimée en utilisant une hypothèse du nombre de leucocytes de 8000 leucocytes par ml de sang.

Extraction des acides nucléiques et synthèse des cDNA

L'ADN génomique (gDNA) des parasites a été extrait en utilisant le kit d'extraction QIAamp DNA Blood kit, selon les recommandations du fournisseur (Quiagen). Les gènes *msp1* et *msp2* ont été amplifiés par la technique Nested PCR en utilisant des amorces spécifiques décrites dans les précédents travaux (185). La multiplicité des infections a été déterminée pour chaque échantillon.

L'extraction des ARN totaux a été réalisée à partir des culots globulaires conservés dans du Trizol selon les recommandations du fournisseur (Invitrogen). Les ARN extraits ont été traités par la DNAse I (Invitrogen) pour enlever d'éventuels contaminations par du gDNA. La synthèse des cDNA a été réalisée selon la méthode développée dans les précédents travaux (185,187).

Caractérisation des isolats : expression de var2csa et adhésion à la CSA

Le profile de transcription et le nombre relatif de copie de *var2csa* a été évalué sur les cDNA extraits. Sur la fraction du culot globulaire mise en maturation, l'expression de VAR2CSA en surface des EI par les isolats a été évaluée par cytométrie en flux en utilisant des anticorps

anti-VAR2CSA obtenus par vaccination génétique chez le lapin. Le niveau d'adhérence de ces isolats au CSPG a été évalué par la méthode des récepteurs immobilisés sur boite de pétri. Ces caractérisations ont été réalisées selon les techniques développées dans les précédents travaux (185–187).

Clonage et séquençage

La région couvrant le NTS-ID2a de *var2csa* (PFL0030c) des positions nucléotidiques 1 à 3100 (3100 pb) a été amplifié à partir des cDNA en utilisant la Phusion DNA polymérase à haute fidélité (New England Biolabs). Les amorces Fw 5–ATGGATAAATCAAGTATTGCT–3 et Rv 5–GAACAGTGGAACAAAGAAATAC–3 ont été utilisés dans les conditions de cycle ci-après : 94°C pendant 1 min, suivie de 35 cycles de 94°C pendant 30 s, 50°C pendant 30 s et 68°C pendant 3 min 40 s, avec une extension finale à 68°C pendant 10 min. Les produits d'amplification ont été soumis à une électrophorèse puis purifiée en utilisant le kit « PCR clean-up, gel extraction » (MACHEREY-NAGEL), ligaturé dans le plasmide pCR™-Blunt II-TOPO® - Vecteur TOPO et transformé au sein des bactéries compétentes One Shot en utilisant le kit de clonage TOPO – Zero Blunt selon les recommandations du fabriquant (Invitrogen). Toutes les colonies ont été analysées pour sélectionner les plasmides contenant l'insert NTS-ID2a par amplification avec le « TEMPase Hot start DNA polymerase » en utilisant les amorces universelles M13F/M13R. Dix 10 clones par échantillon ont été sélectionnées pour générer les séquences, en utilisant trois amorces intermédiaires (A1 : 5-GACAAAAGGTGTGGGAAGTT-3, A2 : 5-ATTGTGTTCTTGGGGG-3 et A3 : 5-TCGTTTCGCGTCTTCTAT-3) en plus des amorces universelles M13 à GATC Biotech (Cologne, Allemagne).

Analyse des séquences

La qualité de tous les chromatogrammes a été vérifiée et les séquences ont été assemblées en utilisant le logiciel DNA Dragon (Sequentix). Les séquences nucléotidiques ont été analysées par BioEdit 7.1 pour identifier les différents clones et identifier leur similarité. Sur une moyenne de 3200 nucléotides par séquence, une différence de clone a été définie sur plus de 10 nucléotides (99%) différents à la même position. Les séquences ont été traduites en acides aminés et alignées en utilisant ClustalW avec les paramètres par défaut, suivie par des corrections manuelles. L'alignement multiple des séquences d'acides aminés en tant que matrice a été généré en utilisant MAFFT version 7 (188). Le score d'entropie, qui représente

une approche robuste permettant la mesure de la variation des séquences (189), a été utilisé pour évaluer le degré de variation d'acides aminés à chaque position d'après l'alignement multiple des séquences en utilisant la formule : $S_{\text{entropy}} = -\sum_{i=1}^{20} p_i \log_2(p_i)$ (*pi* est la fréquence observée des résidus de type *i* dans la colonne alignée). L'entropie de Shannon, à chaque position de l'alignement a été calculée dans BioEdit et tracée en utilisant GraphPad Prism version 5.0**.** La recherche de motifs protéiques associés à certains des paramètres cliniques et parasitologiques étudiés a été réalisée avec le logiciel SigniSite 2.1 (190) pour les phénotypes quantitatifs (âge gestationnel, densité parasitaire, niveau d'adhérence à la CSA, ratio MFI, nombre de copie relatif de *var2csa*) et avec Speer-Server (191) pour les phénotypes qualitatifs (parité avec deux modalités : primigestes *vs* multigestes, et niveau d'adhérence à la CSA avec deux modalités : forts adhérents *vs* faible adhérents). Les valeurs des paramètres associées à un isolat parasitaire donné ont été attribuées à chacune des séquences distinctes de NTS-Id2a identifiés dans cet isolat. Etant donné le grand nombre de positions testées, une correction de Bonferroni a été appliquée pour tenir compte de la multiplicité des tests. Les arbres phylogénétiques ont été générés avec MEGA 5.2 (192) selon la méthode statistique Neighbor-Joining et en utilisant la méthode du test des bootstrap et le modèle de substitution basé sur la distance.

Résultats

Caractéristique des femmes enceintes et des isolats

Les isolats parasitaires provenant de 45 femmes enceintes ont été sélectionnés dans ce travail. Parmi ces femmes, 11 (24%) sont des primigestes. La médiane de la parité de ces femmes est de 2,00 (Interquartile ou IQR, 1 – 3,25). L'âge gestationnel moyen observé est de 21,81. Le niveau moyen d'adhérence à la CSA est de 129,8 parasites/mm^2. La parasitémie moyenne observé dans le sang périphérique des femmes est de 29028,2 parasites par µl de sang. Les autres caractéristiques ont été présentées dans le tableau 1.

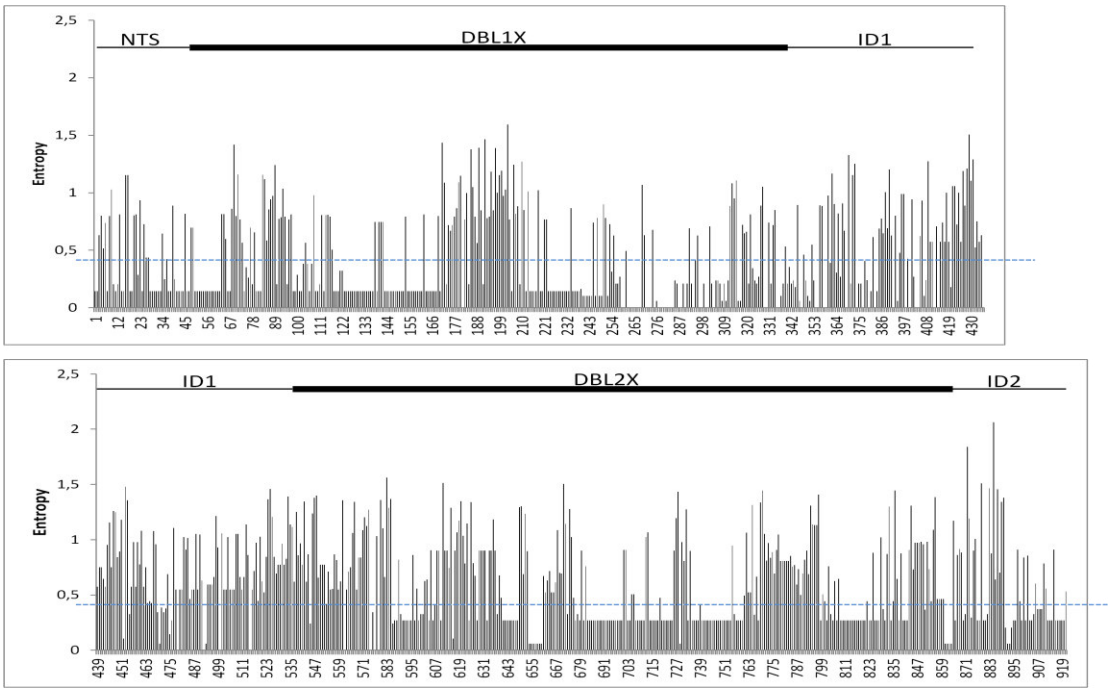
Tableau1 : Quelques caractéristiques des femmes et des isolats

	Moyenne	Médiane	IQR	Etendu
Densité parasitaire (parasites/µl de sang)	29028,2	4224,5	624,25 - 30249,3	0 - 224000
Age gestationnel (semaines d'aménorrhée)	21,8	21,5	14,25 - 30,4	5 - 40.3
Parité	2,6	2	1 - 3,25	1.0 - 8.0
Niveau d'adhérence à la CSA (Parasites/mm^2)	129,8	63	2 - 161	0 - 605
Ratio MFI*	2,2	2,37	1,13 - 3,8	0.29 - 9.14

*Rapport entre la médiane de l'intensité de fluorescence (MFI) observé avec les IgG purifiés des sera de lapins prélevés avant vaccination et le MFI obtenu en marquant la surface des EI par des IgG après vaccination avec toute le partie extracellulaire de VAR2CSA.

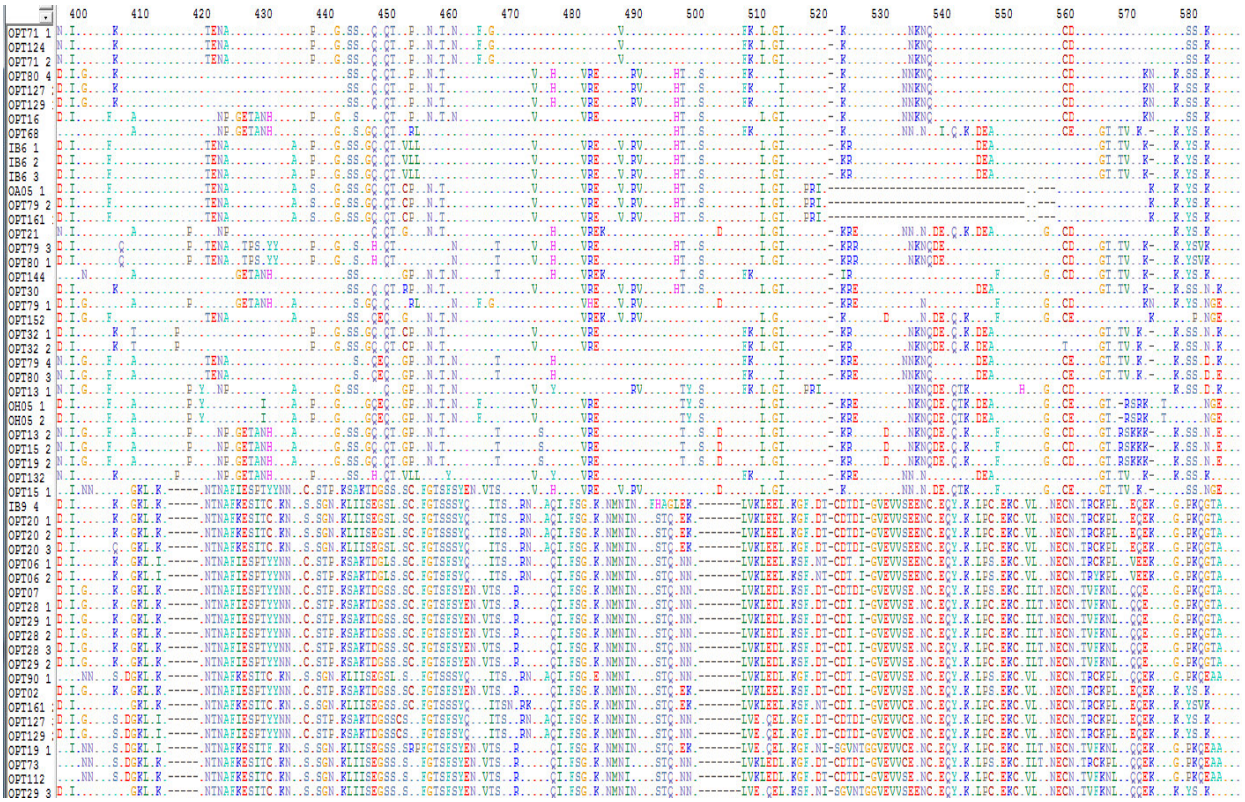
Diversité des séquences du NTS-ID2a

Au total, 398 séquences de NTS-ID2a ont été obtenues des 45 isolats. Parmi ces séquences, 92 sont distinctes au niveau protéique avec une moyenne de 2,06 séquences/isolats. La diversité moyenne observée par paire de nucléotides (π) est de 0,2. La prévalence moyenne des séquences de clones les plus fréquents dans chaque isolat est 50%. Aucune association n'a été établie entre le nombre de séquences distinctes transcrites par les isolats, la parité des femmes, leur âge gestationnel, la densité parasitaire et le niveau de marquage en surface de VAR2CSA. Le niveau de variabilité des acides aminés à chaque site résiduel quantifié par le score d'entropie de Shannon a été présenté dans la Figure 6. La médiane de distribution de H est 0.44 (représentée en trait discontinu dans la Figure 6) et les régions avec une valeur d'entropie élevée correspondent aux régions fortement variables.



<u>Figure 6</u> : Variabilité des séquences d'acides aminés dans la région N-terminale de VAR2CSA.

Pour mieux étudier la variation des séquences dans le segment N-terminal de VAR2CSA, le polymorphisme du NTS-ID2a a été analysé. L'alignement des séquences du segment NTS-ID2 a montré des motifs de séquences conservés entrecoupés de motifs variables. Une région relativement large entre DBL1X et DBL2X met clairement en évidence un dimorphisme de séquences d'acides aminés entre les positions 408 et 584 (Figure 7).

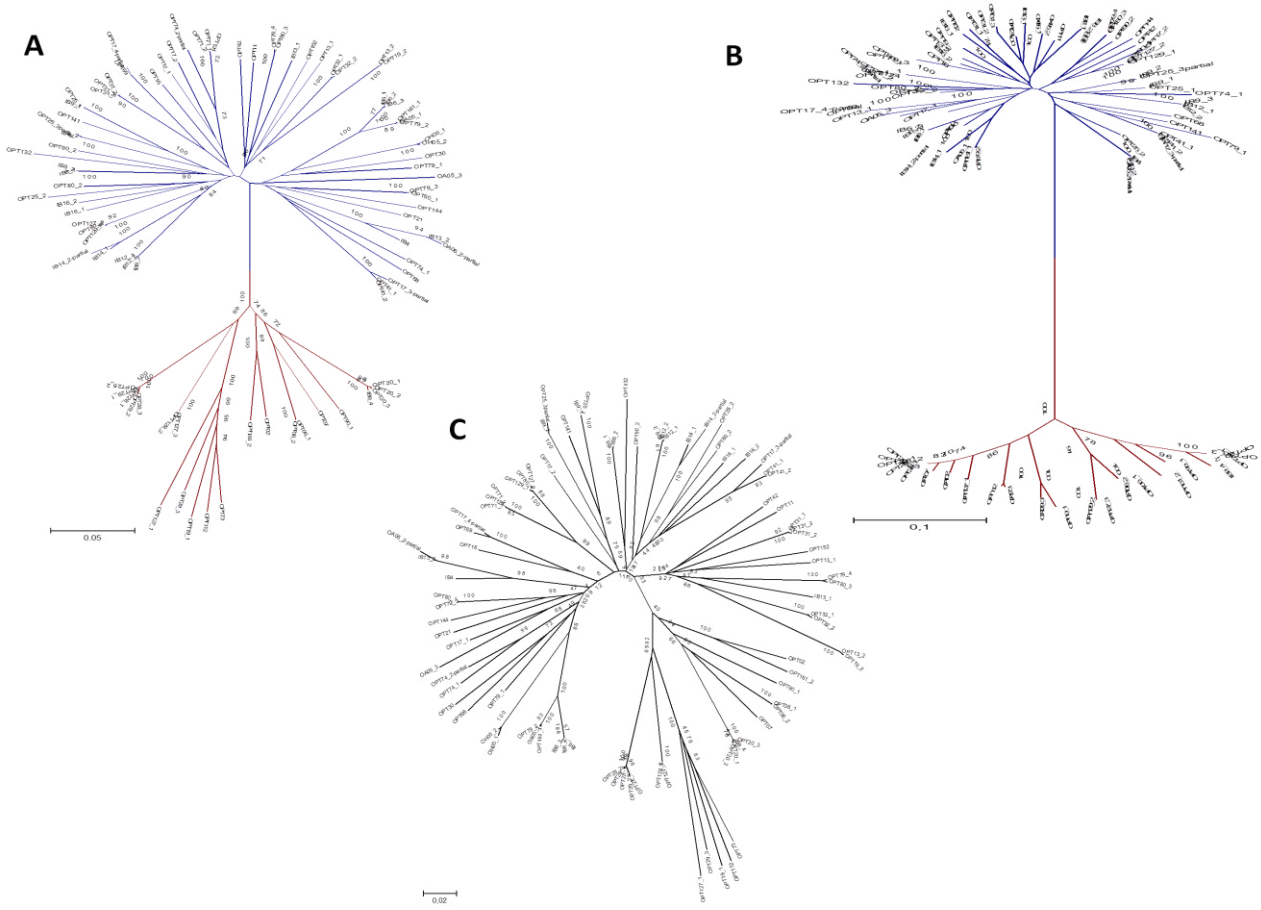


<u>Figure 7</u> : Illustration du dimorphisme des séquences de VAR2CSA entre les positions 408 et 584 de la région ID1

Relations phylogénétiques entre les séquences du segment NTS-DBL2X.

L'analyse phylogénétique réalisée sur les séquences a permis de générer des arbres dont certaine branches sont plus longues que d'autres. Ceci traduit la distance entre les taxons qui est basée sur l'indice de similarité des séquences selon la méthode p-distance et traduit le nombre de différence d'acides aminés par site. Le dimorphisme des séquences du segment NTS-ID2a a été étudié grâce à un standard d'analyse phylogénétique basée sur l'hypothèse selon laquelle les séquences évoluent en grande partie par des phénomènes de mutations. Bien que le segment NTS-ID2a ne soit pas une région hypervariable (64% de similarité entre les séquences et π = 0,2), il présente toutefois des régions très variables notamment dans l'inter domaine ID1 (54,8% de similarité entre les séquences et π = 0,37). La figure 8 montre la construction d'arbres phylogénétiques basée sur 92 séquences du segment NTS-ID2a et de l'inter-domaine ID1 de VAR2CSA obtenu des isolats de *P. falciparum*. La figure 8A montre que ces séquences NTS-ID2a se divisent en deux sous-groupes phylogénétiques distincts (valeur bootstrap 100). Les séquences de 17 variants de VAR2CSA, correspondant à cette région et disponible en ligne (79), ont été associées à l'analyse phylogénétique (Données non

présentées). Il ressort que seul le WR80 appartient au cluster 2 tandis que les variants FCR3, 3D7, HB3 et autres sont regroupés dans le cluster 1. Les arbres correspondant à la région NTS, aux domaines DBL1X et DBL2X et aux inter-domaines ID1 et ID2a ont été également générés pour étudier cette dichotomie des séquences. L'analyse de ces arbres confirme que le dimorphisme est porté par la région ID1 de VAR2CSA (Figure 8B). Pour rechercher si cette dichotomie est due au dimorphisme des séquences dans la région ID1 de VAR2CSA ou à d'autres variations moins évidentes, les séquences ont été re-analysées après excision de l'inter domaine ID1 (Figure 8C). L'arbre généré montre que la division des séquences en deux groupes disparaît et la ramification de l'arbre est similaire à la relation observée entre les variantes d'autres domaines DBL (Données non présentées).



<u>Figure 8</u> : Relations phylogénétiques entre les 92 séquences NTS-ID2a de VAR2CSA.

Les arbres phylogénétiques représentent les séquences correspondant au NTS-Id2a (A), ID1 (B) et à l'excision de ID1 dans le NTS-Id2a (C) Les séquences tombent dans l'un ou l'autre des clusters (cluster 1 : branche en bleu ; cluster 2 : branche rouge)

Relation entre le polymorphisme du NTS-ID2a et le phénotype des parasites.

Pour mieux investiguer sur l'influence du dimorphisme des séquences du domaine ID1 dans le phénotype des parasites et son association avec certains paramètres liés aux femmes infectées, les tests de comparaison multiple ont été réalisés. Le Tableau 2 présente les résultats d'une analyse de comparaison des parasites appartenant aux deux clusters pour les différents phénotypes: parasitémie, l'âge gestationnel des femmes, la parité des femmes, le niveau d'adhérence des parasites à la CSA, le niveau de marquage en surface de VAR2CSA et le nombre relatif de copie de *var2csa*. Bien qu'on observe une certaine tendance vers une adhérence à la CSA plus forte, un niveau de marquage en surface de VAR2CSA plus élevé et un nombre relatif de copie de *var2csa* plus élevé au profit des isolats contenant les clones du cluster 2, aucune différence significative n'a été observée. Toutefois, un effet significatif avec la parasitémie a été révélé. Les isolats portant des clones du cluster 2 présentent une plus forte densité parasitaire comparativement aux isolats n'appartenant pas à ce cluster (*P = 0,03*, test de Mann-Whitney).

Tableau 2 : Comparaison des phénotypes des parasites appartenant aux deux clusters

	Cluster1		Cluster2		
	Moyenne	**Déviation standard**	**Moyenne**	**Déviation standard**	**p-value**
Densité parasitaire (parasites/µl de sang)	12112,2	18433,2	57674,8	73361,0	0,03
Age gestationnel (semaines d'aménorrhée)	23,8	10,2	20,1	9,5	0,27
Parité	2.5	1,8	3,2	2,0	0,16
Niveau d'adhérence à la CSA (Parasites/mm^2)	120,6	151,4	203,8	229,5	0,45
Ratio MFI*	2,4	1,4	3,4	2,6	0,38
Nombre relatif de copie de *var2csa*	6,7	8,6	12,9	32,6	0,79

Recherche des motifs associés aux phénotypes des parasites.

La recherche de motifs avec le logiciel Signisite n'a pas permis de révéler d'association significative entre un motif particulier et la parasitémie (P-values corrigées pour les tests multiples > 0.05). Toutefois, il est intéressant de constater que parmi les 20 sites présentant les résidus les plus associés à la parasitémie (P-values non corrigées pour les tests multiples, comprises entre 0,00017 et 0,0033), 18 d'entre eux (soit 90%) sont situés entre les positions

421 et 573, c'est-à-dire dans la région dimorphique discriminant les deux clusters 1 et 2 (Tableau 3).

Tableau 3 : Positions de la séquence protéique NTS-Id2a présentant la plus forte association avec la parasitémie

Position	Résidu	Sens de l'association avec la parasitémie[a]	Z-score[b]	P-value[c]
713	A	+	3,76	1,73E-04
535	N	-	3,51	4,53E-04
550	A	-	3,38	7,14E-04
541	C	-	3,38	7,14E-04
552	L	-	3,38	7,14E-04
523	N	-	3,38	7,14E-04
530	N	-	3,38	7,14E-04
527	S	-	3,38	7,14E-04
528	S	-	3,38	7,14E-04
529	S	-	3,38	7,14E-04
532	S	-	3,38	7,14E-04
551	S	-	3,38	7,14E-04
558	Y	-	3,38	7,14E-04
533	C	-	3,32	9,00E-04
531	G	-	3,22	1,30E-03
528	*	+	3,04	2,40E-03
806	T	+	3,04	2,41E-03
421	A	-	2,94	3,28E-03
424	A	+	2,94	3,28E-03
422	N	-	2,94	3,28E-03

[a] *Les signes (+) et (-) indiquent que les résidus sont associés à une plus forte ou à une plus faible densité parasitaire, respectivement.*

[b] *Valeur du Z-score calculé par le logiciel Signisite.*

[c] *P-value non corrigée pour les tests multiples.*

** Gap correspondant à une délétion*

Les 18 positions situées dans la région dimorphique du segment ID1 apparaissent en grisé.

La recherche de motifs associés aux autres paramètres analysés a permis de mettre en évidence des positions discriminantes pour deux autres phénotypes :

- Le niveau d'adhérence à la CSA : Quand les isolats sont regroupés en forts et faibles adhérents en considérant la médiane du niveau d'adhérence comme valeur seuil, 4 positions de la séquence protéique (872, 883, 885, 887) sont significativement associées à la capacité d'adhérence des isolats à la CSA (P-values corrigées pour les

tests multiples comprises entre 0.00014 et 0.0497). Bien que les positions soient très rapprochées, le faible déséquilibre de liaison entre elles ne permet pas de distinguer de véritables motifs impliquant plusieurs sites adjacents.

- La parité des femmes (primigestes versus multigestes): Les positions 450, 464 et 466 ont été mises en évidence (P-values corrigées comprises entre 0,00016 et 0,018). Notons que ces positions sont situées dans la région dimorphique de ID1. Une inspection visuelle de la région autour de ces trois positions dans l'alignement multiple des séquences protéiques a permis de révéler trois motifs discriminants dans la région 461-468. Deux d'entre eux (NTHSSIKA et NTHSSIKT) sont significativement plus représentés chez les primigestes (*P = 0.0008*, test exact de Fisher) alors que le troisième (SYENSVTS) est plus fréquent chez les multigestes (*P = 0.0035*, test exact de Fisher) (Tableau 4).

Tableau 4 : Motifs de la séquence protéique NTS-Id2a présentant une association significative avec la parité des femmes enceintes

Motifs dans la région 461-468	Primigestes (*n*=27 séquences)	Multigestes (*n*=55 séquences)	Fisher's exact test P-value	Odds ratio [IC 95%]
NTHSSIK[A/T]	13 (48,1%)	7 (12,7%)	0,0008	6,2 [1.9-22.3]
SYENSVTS	0 (0%)	14 (25,5%)	0,004	∞ [2.0-∞]

IC 95% : Intervalle de confiance à 95% de l'odds ratio.

Discussion et conclusion

L'analyse des séquences du segment NTS-ID2a de VAR2CSA, générées à partir des isolats naturels a permis d'investiguer sur les forces évolutives qui façonnent la variation génétique de *var2csa* dans ce segment spécifique du gène. Ce travail a la particularité d'avoir analysé le polymorphisme de *var2csa* dans un segment contenant le fragment d'intérêt ID1-DBL2X, identifié comme contenant des épitopes protecteurs contre le PG (156, 186,193). Rejoignant les observations antérieures sur la variabilité des séquences d'acides aminés dans VAR2CSA (79), nous montrons l'existence d'une forte variabilité de séquences de VAR2CSA dans certaines régions du segment NTS-ID2a. Les travaux de Sander en 2009 ont mis en évidence un dimorphisme de séquences d'environ 66 acides aminés dans le domaine DBL2X (162). Ces motifs ont également été observés dans les séquences analysées au cours de ce travail. Toutefois elle n'entraine pas une ségrégation nette des séquences du NTS-ID2a lorsque la

région ID1 est excisée. Ceci suggère que le dimorphisme des séquences observé dans la région ID1 est beaucoup plus prononcé que celui présent dans le domaine DBL2X. Nous mettons en évidence dans ce travail, une dichotomie des séquences qui s'étend sur toute la région ID1 et qui conduit à l'indentification de deux clusters distincts. Cette signature moléculaire dans les séquences de VAR2CSA a été décrite pour la première fois dans ce travail.

Les résultats de cette étude permettent de démontrer une association significative entre cette dichotomie des séquences dans la région ID1 de VAR2CSA et les infections à forte densité parasitaire des isolats de *P. falciparum* (P = 0.03). Ceci suggère que les parasites porteurs des séquences ID1 appartenant au cluster 2 seraient les plus virulents avec un taux d'infection des mérozoïtes plus élevé ou présenteraient une meilleure résistance au système immunitaire. Ces caractéristiques augmenteraient le risque de complications lors d'infections à *P. falciparum* par ces clones pendant la grossesse. Les analyses sont actuellement en cours pour mieux explorer la distribution géographique de ce génotype de VAR2CSA à partir de séquences d'isolats parasitaires provenant d'autres régions géographiques et évaluer son association avec les motifs du domaine DBL2X liés à une hyper-parasitémie (194).

En dépit d'une tendance indiquant des valeurs plus élevées des autres paramètres étudiés, tels que le niveau d'expression de VAR2CSA et le niveau d'adhérence à la CSA, pour les parasites portant des séquences du cluster 2, nous n'avons pas mis en évidence d'association significative entre ces paramètres et le cluster d'appartenance. Ceci peut s'expliquer par le petit nombre d'isolats étudiés (31 du cluster 1 et 14 du cluster 2) qui confère une faible puissance statistique. Toutefois, la recherche des motifs discriminatifs de ce dimorphisme dans la région ID1 de VAR2CSA a permis de montrer une nette association avec la parité. Une étude réalisée sur un effectif d'isolats plus large devrait permettre de mieux analyser l'impact de cette signature moléculaire dans la région ID1 de VAR2CSA sur le phénotype des isolats et investiguer sur les propriétés des anticorps dirigés contre les variants de VAR2CSA portant cette signature dans la région ID1.

Partie IV : Discussion générale et conclusion

Le facteur majeur dans la pathogenèse du paludisme gestationnel réside dans la capacité des EI par les formes matures de *P. falciparum*, à être séquestré dans le placenta. Cette séquestration est attribuée à l'interaction entre les protéines parasitaires exprimées à la surface des EI et les récepteurs placentaires localisés sur les syncytiotrophoblastes. L'intérêt majeur de cette thèse a donc été de générer davantage d'informations sur les caractéristiques génotypiques et phénotypiques des parasites infectant les femmes enceintes et donc responsables de la séquestration placentaire.

Il est clairement établi que l'expression différentielle des groupes ou sous-groupes du gène *var* chez *P. falciparum* est associée à la physiopathologie du paludisme. Pour explorer d'avantage cette question dans le contexte particulier du PG, nous avons caractérisé le profil d'expression des gènes *var* par des isolats de *P. falciparum* collectés chez les femmes gestantes. Les résultats montrent que de nombreux transcrits de gènes *var* distincts étaient détectables dans la majorité des isolats étudiés dans ce travail. Il apparait clairement que des gènes *var* appartenant aux groupe A, B, C et D dont l'expression a été associée à d'autres formes cliniques du paludisme (66,67,68,195) sont également transcrits par des isolats infectant les femmes enceintes (143). Mais l'analyse de l'abondance de chaque transcrit identifié révèle de nettes différences entre les isolats parasitaires analysés et ce, en fonction de la forme clinique du paludisme comme attendu. Nos données confirment les résultats déjà publiés et montrent que les parasites de femmes enceintes transcrivent fortement *var2csa* quelle qu'en soit leur provenance (sang périphérique ou placentaire) et le moment de l'infection au cours de la gestation (premier trimestre de grossesse, deuxième trimestre ou à l'accouchement) (62,70,143,168,196,197). Le gène *var* dominant, transcrit au cours d'un cycle parasitaire donné, devrait être préférentiellement traduit en protéine PfEMP1, médiatrice d'un phénotype d'adhérence particulier. Nous avons démontré la présence de VAR2CSA à la surface de la majorité des EI provenant de femmes enceintes dont les parasites transcrivaient préférentiellement le gène correspondant. Ces observations corroborent celles des travaux antérieurs sur la transcription de *var2csa*, et mettent en évidence les caractéristiques phénotypiques particulières des parasites qui infectent la femme pendant la grossesse. Des études antérieures ont montré que des anticorps spécifiques des AVS exprimés par des parasites peuvent déjà être identifiés dans le plasma des sujets donneurs de parasites au moment de la collecte (198,199). Nos données non seulement confirment la présence des anticorps spécifiques de VAR2CSA, comme un marqueur d'infections chez les femmes enceintes, mais confortent aussi l'hypothèse selon laquelle *var2csa* joue un rôle majeur dans

la pathogenèse du PG. Ces résultats rejoignent les observations des travaux antérieures réalisés chez les femmes enceintes incluses à l'accouchement (70,200), et mettent en lumière l'association de cette signature aux infections survenant à différents moments de la grossesse.

L'aspect fonctionnel de VAR2CSA exprimée par les parasites du PG, défini par la capacité des EI à adhérer aux récepteurs placentaires CSA a été analysé dans ce travail. Plusieurs études ont décrit le phénotype d'adhérence qui caractérise les parasites recueillis auprès des femmes pendant la grossesse (9,11,141), en démontrant la capacité de ces isolats à adhérer spécifiquement à la CSA. Cependant la plupart de ces études a porté sur des isolats obtenus à l'accouchement. Les phénotypes d'adhérence des parasites infectant les femmes enceintes en début de grossesse et leur dynamique tout au long de la grossesse restaient mal connus.

Bien que les données d'études antérieures aient montré que les anticorps anti-VAR2CSA sont déjà présents au deuxième trimestre de grossesse chez la plupart de primigestes, suggérant fortement l'infection avec des parasites exprimant VAR2CSA (78,107), nous avons pu montrer dans ce travail que la majorité des parasites causant des infections en début de grossesse expriment déjà VAR2CSA et adhèrent à la CSA. Le fait que les femmes en début de grossesse (dès la 7ème semaine de gestation) peuvent abriter des parasites *P. falciparum* exprimant VAR2CSA et adhérant à la CSA suggère que l'irrigation placentaire par le sang maternel se produirait beaucoup plus tôt qu'on ne le pensait (201,202). Bien que le mécanisme impliqué reste à être élucidé, ces observations soulignent la nécessité d'aborder la question de la prévention du paludisme en début de grossesse pour prévenir l'infestation du placenta. Ce besoin urgent est étayé par des études récentes qui montrent un impact des infections survenant dans le premier trimestre sur le bon déroulement de la grossesse, en affectant à la fois la mère (anémie maternelle) et le nouveau-né (faible poids de naissance) (203,204).

Une analyse élargie des phénotypes d'adhérence des parasites de femmes enceintes sur d'autres récepteurs connus comme CD36 et ICAM-1, met en évidence la coexistence de parasites avec plusieurs phénotypes d'adhérence en début de grossesse. Cette complexité de phénotype à un moment où le placenta n'est pas encore bien établi suggère que la femme à ce stade de la grossesse est susceptible aux infections pouvant survenir chez des adultes vivant en région d'endémie palustre. Il est donc probable que l'immuno-modulation généralisée qui se produit au cours de la grossesse favorise les infections par *P. falciparum* indépendamment du phénotype d'adhérence des parasites. Mais cette diversité phénotypique observée se resserre progressivement avec l'âge gestationnel en faveur du phénotype d'adhérence à la

CSA. Ceci suggère que dès la mise en place des moyens de prévention et dès lors que la femme se présente en consultation prénatale, par les conseils sanitaires, l'initiation du TPI, la structure des populations parasitaires va être considérablement modifiée pour donner lieux à l'expansion des parasites moins connus du système immunitaire, en l'occurrence sélectionnés par les récepteurs placentaires. Ceci souligne le risque élevé d'infection des femmes par des parasites CSA-adhérent, comme déjà décrit dans des études antérieurs réalisées en fin de grossesse (10,115,119) et suggère leur plus grande implication dans la pathogenèse du PG, Dans ces études, les interactions des EI avec CD36 et ICAM- 1 ont été rapportées avec quelques isolats provenant de femmes enceintes. Toutefois, comme dans notre étude l'adhérence à la CSA a été le phénotype le plus observé chez les isolats de femmes enceintes et avec généralement des niveaux plus élevés par rapport au CD36 et ICAM-1.

Dans ce travail, la préférence d'adhérence des EI à la CSA a été exclusivement observée parmi les isolats chez qui la transcription de *var2csa* est clairement dominante par rapport à celle des autres gènes *var* (196,197). La co-expression de plusieurs gènes *var* serait l'œuvre de la variation phénotypique clonale propre à *P. falciparum* et à la multiplicité des infections, certains isolats abritant plusieurs clones parasitaires. Ceci expliquerait la capacité d'adhérer à plus d'un récepteur observée chez certains isolats. Ces observations indiquent clairement que les infections par des parasites n'adhérant pas à la CSA se produisent également au cours de la grossesse. Bien que l'importance de ces infections dans l'issue de la grossesse soit encore mal connue, leur caractérisation reste une question ouverte. Ceci suggère tout de même la nécessité d'une intégration de plusieurs moyens de préventions dans le cadre de la bonne prise en charge de la grossesse dans les régions où le paludisme est endémique. La restriction du phénotype d'adhérence des parasites ayant une préférence pour la CSA se produirait progressivement à mesure que le placenta se développe et devient de plus en plus irrigué. D'autre part, les phénotypes d'adhérence aux récepteurs non-CSA semblent être plus fréquents chez les multipares, chez qui l'immunité contre les parasites à tropisme placentaire est bien décrite (158). Cette réémergence des infections par ces parasites non-CSA spécifiques suggère un meilleur contrôle des phénotypes d'adhérence à la CSA via l'immunité acquise, rétablissant ainsi la diversité des propriétés d'adhérence observées en début de grossesse, en particulier chez les femmes primigestes.

De nombreuses études ont démontré la grande vulnérabilité des primigestes au PG en raison de l'absence d'immunité protectrice qui est acquis au cours de grossesses successives (6), (158,184). Corroborant les observations de ces travaux, nos données soulignent la grande

vulnérabilité des femmes primipares face à l'infection par des parasites exprimant un phénotype d'adhérence à la CSA. Le fait que les parasites provenant des primigestes adhèrent plus fortement à la CSA que ceux des multigestes suggère que ce facteur est la cause majeure des conséquences généralement plus marquées chez les primigestes.

Plusieurs travaux ont permis de construire l'hypothèse selon laquelle, l'acquisition des anticorps dirigés contre VAR2CSA serait un facteur de résistance et de protection contre le PG. Au cours de notre travail, nous avons mis en évidence l'existence chez les femmes enceintes d'IgG dirigées contre des antigènes de VAR2CSA, dont la spécificité et le niveau varient avec la parité des femmes. Par ailleurs, nous montrons que ces anticorps produits contre VAR2CSA sont aussi présents chez des individus non-enceints, bien que le niveau et la prévalence de ceux-ci reste inférieur par rapport aux femmes multigestes exposées à *P. falciparum* (205–207). Cette observation s'expliquerait par le fait que VAR2CSA est également détectable chez des parasites d'origine non-placentaire (208), même si son expression n'est pas prédominante, il peut induire des réponses transitoires chez certains sujets. Mais ces anticorps, pour la plupart, ne peuvent efficacement inhiber l'adhérence des isolats à la CSA. Les différents travaux ayant abordé ce sujet ont presque tous été réalisés en Afrique subsaharienne région portant le plus lourd fardeau du paludisme gestationnel. La Colombie est aussi endémique au paludisme et présente une caractéristique épidémiologique du paludisme différente de celle des régions endémiques africaines (209). Nous avons investigué la prévalence des anticorps anti-VARCSA au sein de cette population et avons pu mettre en évidence une séroprévalence élevée d'anticorps anti-VAR2CSA. Contrairement aux études africaines chez des sujets non-enceints, il est apparu que les anticorps observés chez des sujets non-enceints Colombien étaient capables d'inhiber l'adhérence à la CSA des souches CSA-adhérents dans les populations colombiennes en dehors et pendant la grossesse. Cette observation pourrait résulter de la co-circulation de *P. falciparum* et *P. vivax,* et suggère l'existence d'épitopes semblables, en partage chez ces deux espèces. Une autre hypothèse repose sur des différences génotypiques ou phénotypiques entre les souches exprimant VAR2CSA en Colombie et les parasites rencontrés en Afrique. Ces parasites peuvent ainsi être caractérisés par une variation de séquence dans le gène *var2csa*, ou une modification des mécanismes d'expression des protéines VAR2CSA, de régulation et de l'immunogénicité en faveur d'une expression de *var2csa* en dehors de la grossesse. Des études supplémentaires sont à prévoir pour caractériser le profil transcriptionnel des gènes *var* et de cytoadhérence

des isolats de *P. falciparum* circulant dans d'autres régions géographiques, en dehors de l'Afrique, où d'autres espèces de Plasmodium circulent en plus de *P. falciparum*.

Ce travail de thèse a contribué à l'identification de la région N-terminale de VAR2CSA qui contient des épitopes capables d'induire des anticorps anti-adhérence à large spectre d'activité. Nous avons utilisé dans notre étude la technologie de vaccination génétique qui s'est avérée efficace sur divers agents pathogènes et des antigènes tumoraux (210). Cette technique a été appliquée avec succès dans nos travaux sur le gène *var2csa* de *P. falciparum*. L'utilisation de cette technique simple permet d'éviter l'étape longue et difficile de la production de protéines recombinantes. Il est alors beaucoup plus rapide et plus facile à mettre en œuvre pour filtrer et comparer différentes séquences codantes par leur immunogénicité. En outre, la précision moléculaire offerte par l'immunisation à base de gènes est très attractive, tandis que les protéines recombinantes sont souvent porteuses de tags qui sont de courtes séquences supplémentaires nécessaires pour leur purification. Nous avons généré des arguments qui soutiennent le fait que la construction antigénique correspondant au NTS-DBL2X ou contenue dans ce fragment, concentrerait les épitopes à même d'induire des anticorps avec une capacité d'inhibition de l'adhérence des EI similaire à celle obtenue avec la totalité de la partie extracellulaire de VAR2CSA. De plus, en combinant les propriétés d'inhibition de l'adhérence de cette construction ciblant les variants FCR3 et 3D7, on augmente sensiblement la proportion d'isolats de parasites inhibés par les anticorps induits par vaccination. D'une part, cela met clairement en évidence l'existence d'une fonction importante des épitopes dans cette région de VAR2CSA, qui sont partagés par plusieurs isolats de *P. falciparum*, séquestrant dans le placenta. D'autre part, ces observations supposent que les quelques variations de séquence de VAR2CSA pourraient être critiques et avoir une implication fonctionnelle sur la protéine exprimée par les isolats de terrain. Les données de ce travail, bien que produite sur un petit nombre d'échantillons, suggèrent clairement qu'une proportion plus élevée d'isolats parasitaires serait ciblée par une approche de vaccin multivalent comprenant au moins deux génotypes de VAR2CSA dans sa partie d'intérêt, à savoir dans le fragment NTS-DBL2X. Ces observations sont d'une importance majeure dans l'effort continu visant à créer un vaccin optimal à base de VAR2CSA. Cette hypothèse mérite donc d'être consolidée par des travaux similaires sur un échantillonnage plus large.

Par ailleurs, les données présentées dans ce travail indiquent clairement, que l'expression en tandem de ID1 est une nécessité fondamentale pour l'induction d'anticorps anti-adhérence.

Ces observations suggèrent que ID1 est un composant fondamental dans la conformation de l'épitope capable d'induire des anticorps anti-adhésion au placenta. Il apparaît dès lors que les variations de séquence dans la région ID1 de VAR2CSA pourraient influencer la fonctionnalité des anticorps dirigés contre les constructions incluant ID1. Une composante majeure de ce travail s'est penchée sur cette hypothèse en analysant les séquences du fragment N-terminale de VAR2CSA sur plusieurs isolats caractérisés pour leur phénotype d'adhérence. Le but étant d'identifier des variations pouvant influer l'acquisition d'anticorps anti-adhérence. Nous mettons clairement en évidence un dimorphisme dans le domaine ID1 qui permet de ségréger des parasites en deux groupes bien distinct. Cette dichotomie de séquences dans cette région conduit à une ségrégation des isolats et l'identification des motifs de discrimination qui recouvrent ~168 acides aminés. Une telle signature moléculaire nette et bien marquée a rarement été décrite sur VAR2CSA. Dans des travaux précédents de notre équipe, une telle signature a été rapportée sur le domaine DBL2X, mais son implication dans la fonctionnalité de la protéine reste non prouvée. L'un des clusters définis dans le segment NTS-ID2a contient les séquences caractéristiques des souches FCR3 et 3D7 à partir desquelles plusieurs études de caractérisation d'anticorps protecteurs ont été réalisées à ce jour. L'autre cluster est quand à lui caractéristique des isolats rarement rencontrés en Asie du Sud-Est et n'ayant pas encore été documentés en Afrique (79). De façon très intéressante il apparaît que les parasites portant cette nouvelle signature dans la région ID1 sont associés à des infections donnant lieu à de fortes parasitémies. Il est établit que la densité parasitaire est un facteur de gravité majeur du paludisme gestationnel. L'identification de ce cluster dans notre région d'étude est d'un grand intérêt dans l'étude de la diversité de la population de *P. falciparum* infectant la femme enceinte et la compréhension de la pathogenèse du PG. Les isolats exprimant ce variant Id1 de VAR2CSA associé à une forte parasitémie, seraient donc potentiellement, les plus pathogènes et augmenteraient éventuellement, le risque d'une issue défavorable de la grossesse. Des motifs de séquences dans les domaines DBL2X et DBL3X de VAR2CSA ont également été associés à une hyperparasitémie dans le cadre du paludisme placentaire (194). Ces observations sont pertinentes pour comprendre les mécanismes moléculaires impliqués dans la séquestration placentaire des parasites, ainsi que pour le développement de nouveaux outils de prévention contre le paludisme placentaire. La caractérisation de ces parasites qui expriment ces signatures à travers leur capacité d'adhérence à la CSA, leur profil de transcription des gènes *var* et leur comportement face aux anticorps fonctionnels sont des questions qui devront être évaluées à l'avenir.

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